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BIOLOGICAL EFFECTS OF LASER RADIATION

Final Scientific Report - Volume III
(Effects of Carbon Dioxide Laser Radiation on the Rabbit Eye)

Donald Lewis MacKeen
Samuel Fine

Submitted October 17, 1978
(1 July 1963 to 30 September 1971)

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Department of Biophysics and Biomedical Engineering
Northeastern University
Boston, Massachusetts 02115

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Effects of carbon dioxide laser irradiation of the cornea on the anterior segment of the rabbit eye were studied. Corneal temperature elevations were correlated with corneal tissue changes. The average steady state corneal surface temperature at CO_2 laser irradiation levels considered to be safe (100 mW/cm^2) was 5.5°C, at 200 mW/cm^2, where irreversible injury occurs, it was less than 10°C, and at 380 mW/cm^2 it was less than 15°C. At 100 mW/cm^2, there was essentially nc elevation in intraocular pressure; at 350 mW/cm^2; elevation was 20 mm Hg. A transient increase in aqueous temperature and pressure occurred on pulsed corneal

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irradiation. Anterior lenticular indentation, observed on fixation in glutaraldehyde, was determined as due to concomitant anterior chamber temperature and pressure elevation. Indentation was probably due to lens surface temperature in excess of 60°C together with anterior chamber pressure increase. Lens indentation following glutaraldehyde fixation was associated with corneal flattening. Aqueous protein concentration was maximum following irradiation over the center of the iris. Alterations in lens glutathione, ascorbic acid and soluble lens proteins occurred on suprathreshold irradiation. The glutathione decrement was not due to increased conversion to oxidized glutathione, but was coincidental with alterations in soluble lens protein electrophoretic mobilities.

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Note on Content

This volume contains essentially the material submitted to Northeastern University as a Ph.D. thesis in Biology in July 1972.

The University doctoral committee consisted of Drs. Charles Gainor, Charles Goolsby, John Reinhard, Elliott Spector, Brunhild Stuerckow and Helen Lambert. The external readers were Drs. Herbert Berman, Ben Fine and Jin Kinoshita.

The thesis was supervised by the Principal Investigator. Much of the work detailed here was supported under this contract.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

Technical Acknowledgement

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ABSTRACT

The effects of carbon dioxide (CO_2) laser irradiation of the cornea on the anterior segment of the rabbit eye have been investigated. Corneal temperature elevations have been correlated with previously reported corneal tissue changes. The average steady state corneal surface temperature at CO_2 laser irradiation levels considered to be safe (100 mW/cm^2) was determined to be 5.5°C, at 200 mW/cm^2, where irreversible injury occurs, it was less than 10°C, and at 380 mW/cm^2 it was less than 15°C. On continuous corneal irradiation at 100 mW/cm^2, there was essentially no elevation in intraocular pressure; at 350 mW/cm^2, there was an elevation of 20 mm Hg. Pulsed irradiation of the center of the cornea resulted in a transient increase in aqueous humor temperature and in aqueous humor pressure. Anterior lenticular indentation, which was not evident on dissection but observed on fixation in glutaraldehyde, was determined as due to concomitant temperature and pressure elevations in the anterior chamber. The lens indentation was probably due to a combination of temperature at the lens surface in excess of 60°C together with a rise in pressure in the anterior chamber. The temperature and pressure elevations necessary to cause lens indentation following glutaraldehyde fixation were associated with corneal flattening. On spectroscopic determination of aqueous protein in these studies, the concentration was maximum following irradiation over the center of the iris. Delayed alterations in lens glutathione, ascorbic acid and soluble lens proteins occurred on suprathreshold irradiation of the corneas of weanling rabbits. The decrease in ascorbic acid has been observed by others on irradiation

at specific wavelengths in the electromagnetic spectrum. The lens glutathione decrement was probably not due to an increased conversion to oxidized glutathione, and was coincident with an alteration in electrophoretic mobility observed in soluble lenticular proteins. This decrease in electrophoretic mobility was greatest in the β and σ crystallins.

These results provide information regarding the mechanism of injury on accidental ocular exposure to CO_2 laser radiation, and consequently should permit better management of accidents. Furthermore, the findings show that the CO_2 laser can be used as a tool both for the investigations of the responses of ocular tissue to controlled heating and for obtaining basic information regarding the anterior segment of the eye.

FOREWORD

The electromagnetic radiation emitted by the carbon dioxide (CO_2) laser at 10.6 u is potentially harmful to all biological material. The potential hazard from this radiation results from the combination of collimated high power density (i.e., power intensity) outputs available, the low absorption of the radiation by the atmosphere, the large beam reflectivity by metal surfaces and the high absorptivity of tissues. The anterior segment of the eye is especially vulnerable to damage from this radiation because of its exposed position plus the functional requirement of the tissues along the visual axis to remain transparent.

The investigation of the physical and biological occurrences during deliberate quantitated laser injury of tissue should provide an insight into more knowledgeable management of accidental injuries. Furthermore, results of such studies may increase knowledge of the physiology of the tissue investigated.

This research is concerned with the effects of CO_2 laser radiation on biological tissue, in particular, the anterior segment of the rabbit eye.

The first section, introductory in nature, is divided into two parts. The first part is a discussion of the basic aspects of the carbon dioxide laser. The second part provides pertinent information regarding the morphology, physiology and biochemistry of the anterior segment of the rabbit eye, as well as responses of this region to injury.

The second section, Chapter I, is a review of literature concerning the action of CO_2 laser radiation on biological material.

The third section, Chapter II, describes an investigation of the relationship between the power density of the CO_2 laser radiation impinging on the corneal surface and the resultant steady-state elevation of the temperature of the anterior segment of the eye. The relationship between the corneal surface temperature elevation and the threshold for permanent damage on continuous irradiation was determined experimentally. Also, the steady-state corneal surface and anterior chamber temperatures were simultaneously measured over a range of suprathreshold power density irradiations. The findings of this section should enable one to correlate elevations of corneal temperature with resultant tissue damage. Furthermore, the data permit estimation of the steady-state temperature of the anterior chamber by means of a simple corneal surface temperature measurement on CO_2 laser irradiation.

The fourth section, Chapter III, presents an investigation of changes in the anterior segment of the eye during corneal irradiation. Pressure and temperature were measured in the anterior chamber during both continuous suprathreshold power density, as well as pulsed higher power density focused irradiations of the cornea, and their relationship to lens alterations is discussed. This chapter also contains descriptions of the transient flattening of the cornea during irradiation and the resultant decrease in the depth of the anterior chamber. Alterations of the concentration of the aqueous humor protein on irradiation are discussed.

The fifth section, Chapter IV, describes an investigation of the alterations of the gross morphology of cornea, iris, and lens following

continuous relatively high power CO_2 irradiation of the cornea. Concomitant alterations of the electrophoretic pattern of soluble lenticular proteins, and alteration of lenticular ascorbic acid and reduced glutathione were measured.

The sixth section, Appendices, contains a description of the CO_2 laser and associated equipment used in these investigations; the method and equipment used for the electrophoresis of soluble ocular proteins, and methods and techniques employed for the determination of ascorbic acid, and of reduced and oxidized glutathione are discussed.

INTRODUCTION

The term laser is an acronym for Light Amplification by Stimulated Emission of Radiation. Laser output is relatively coherent, monochromatic electromagnetic radiation. A large number of substances are capable of producing laser radiation; their lasing output wavelengths range from the ultraviolet through the visible to the infrared portion of the electromagnetic (EM) spectrum. Lasers are presently available with pulsed power outputs as high as terrawatts (10^{12} watts).

In their reviews of the biological effects of laser radiation, Fine and Klein (1965) reported that the first definitive proposals for utilizing the principle of stimulated emission were made independently by Townes et al., by Weber, and by Basov and Prokhorov. In 1955, Gordon, Zeiger and Townes reported the first successful application of amplification by stimulated emission with an ammonia maser (Microwave Amplification by Stimulated Emission of Radiation). In 1958, Shawlow and Townes proposed extension of this principle to the visible region of the EM spectrum and in 1960 Maiman succeeded in obtaining lasing action from an artificial ruby rod excited with light from a xenon flash lamp. The output of this solid-state laser was a one millisecond pulse in the red (694 nm) region of the spectrum.

The helium neon (HeNe) laser was the first gas laser capable of producing a continuous wave (cw) laser output in the visible portion of the EM spectrum at 632.8 nm.

The carbon dioxide laser was first described by Patel (1965). This molecular laser containing CO_2 and N_2 can produce a continuous

output in the middle infrared portion of the EM spectrum at 10.6μ. Patel, Tien and McFee (1965) reported that the addition of helium to the CO_2 and N_2 increased the laser power output.

Basic Aspects of the CO_2 Laser

The following information was obtained in part from Fine and Klein (1965) and Siegeman (1971). A simplified graphical display of the initial phase of the lasing process for many lasers, and specifically for the CO_2 laser is shown in Figure In-1. The active element of the CO_2 laser is gaseous, in contradistinction to the ruby laser, which is in the solid state. Gaseous CO_2, N_2 and He are electrically excited in a water-cooled glass tube called a plasma tube or chamber. The ordinate of Figure In-1 indicates the energy level; E_0 is the unexcited ground state of the molecule, and E_1 a discrete higher energy level. The electrically stimulated helium molecules collide with and elevate the nitrogen molecules to a higher energy level, E_1. The energy difference between $E_1 - E_0$ for nitrogen is similar to that of carbon dioxide; consequently, the energy is transferred readily from the nitrogen to the carbon dioxide molecule. The nitrogen molecule descends in a series of energy level steps to the ground state.

When more carbon dioxide molecules are at an energy level E_1 than at an energy level E_2, a population inversion is said to exist between these levels.

Initiation of the lasing process occurs when the energy of one molecule drops from the E_1 level to E_2 (Step [2]). The wavelength of

the electromagnetic energy released in this descent is a function of the $E_1 - E_2$ distance in joules in which $\nu = \frac{E_1 - E_2}{h}$, where ν = the frequency in Hertz and h = Planck's constant (6.6×10^{-34} joule-sec). The wavelength in microns, $\lambda = \frac{C}{\nu}$, where C = speed of light (3×10^{14} microns/second).

The initial emission stimulates the descent of adjacent molecules or ions from their elevated energy levels. This is graphically illustrated in Figure In-2, where the photons are added in phase.

This released electromagnetic energy is produced in the plasma tube located between two parallel mirrors; the distance between the mirrors is a function of the emitted radiation wavelength. The large ratio between the mirror distance and the plasma chamber diameter serves to collimate the radiation. The parallel end windows reflect the radiation causing more stimulated emission; if a constant population inversion process occurs, the emitted radiation can be continuous; otherwise, the emitted radiation is in pulses. One of the chamber mirrors is approximately 100% reflective; the reflection of the other is less than 100%. This less reflective window allows a fraction of the total energy in the laser cavity to exit the chamber. The energy which exits the cavity is essentially monochromatic, coherent and, because of the geometry of the laser cavity, collimated. The collimation produces a nearly parallel path of radiation with little beam divergence.

The carbon dioxide laser has been used for a host of applications, both industrial and medical; many units are commercially available for either of these applications. The focused output has been used in

industry to drill, cut or scribe ceramics, metals and many other materials. It has been used experimentally as a "hot" knife in an attempt to carry out essentially blood-free surgical operations and in the removal of certain tumors by ablation. At present, however, it remains only an experimental tool in medicine. Use of the beam in the study of atmospheric pollution has been proposed because of the differential in attenuation of the beam by air and certain atmospheric pollutants.

The Anterior Segment of the Normal Rabbit Eye

In this section we will discuss the specific aspects relating to the anatomy, physiology and biochemistry of the anterior segment of the normal rabbit eye pertinent to these investigations. According to Prince (1964a), the rabbit cornea is a clear structure about 300 u thick in the young, and 500 u in the mature animal, composed of four distinct regions: the outermost layer of epithelium, the underlying stroma, Descemet's membrane, and the innermost layer of endothelial cells. According to Fine and Yanoff (1972), the endothelium of the cornea is a misnomer and might be more accurately termed a mesothelium in that an endothelium is a layer of cells lining a vascular or lymphatic channel, whereas mesothelial cells line body cavities. They cite evidence that the corneal "endothelial" cells can respond pathologically in a manner characteristic of mesothelial cells. To avoid confusion, however, references to this layer will use the conventional term, endothelial. The epithelium is about five cell layers deep. The outermost layer consists of flat

squamous cells. The epithelial basement membrane (bm) is observable best by transmission electron microscopy. Its presence can be readily suspected in light microscopy by the slight periodic acid-Schiff (PAS) positivity which may be observed in this plane (B. S. Fine, personal communication, 1972). Unlike the human cornea, the rabbit cornea does not have an easily recognized, distinctive zone (i.e., Bowman's membrane) between the epithelial layer and the stroma. About 90% of the thickness of the entire cornea is stroma, composed of interlacing bands, each of which is composed of fine collagen fibrils whose axes are roughly parallel to the corneal surface. The fibrils are roughly equally spaced from one another and are surrounded by a mucopolysaccharide containing ground substance. Normally, the stroma is maintained in a relatively dehydrated state. Descemet's membrane is a layer (approximately 10 u thick) separating the posterior surface of the stromal layer from the endothelial cells and is considered to be the basement membrane of the endothelial cells. The single layer of endothelial cells comprise the innermost layer of the cornea.

The anterior chamber is normally filled with aqueous humor ("aqueous"). The chamber is bounded anteriorly by the curved posterior surface of the cornea and posteriorly by the iris and pupillary surface of the lens. The volume of the anterior chamber in the weanling rabbit eye is less than 200 u L and the maximum depth along the visual axis is about 2mm. The adult eye chamber volume is approximately 250 u L and the depth is 3 mm. The anterior chamber aqueous humor is in fluid contact with that of the smaller posterior chamber which contains about one-fifth the

volume of the anterior chamber (Prince, 1964a). The avascular lens and cornea are mainly dependent on the aqueous humor for normal metabolism (Davson, 1969). Apart from its role in metabolism, a normal steady state influx-efflux relationship is necessary to sustain the intraocular pressure at approximately 20 mm Hg above atmospheric pressure. This maintenance of adequate intraocular pressure is necessary in order to maintain an optically smooth arc at the corneal refracting surface.

The entire iris, including the ciliary processes, is well supplied with blood, mainly by branches from the ciliary artery. In the rabbit eye the posterior surface of the iris has approximately 80 radially-distributed structures. These structures have been termed by Kozart (1971) as either iridial or ciliary on the basis of their location. The iridial processes are the more prominent of the two. Many of the iridial processes are in contact with the anterior pre-equatorial surface of the lens (Langley, Mortimer and McCulloch, 1960). In contrast, the ciliary processes of the adult human eye are located on the anterior portion of the ciliary body and usually cease (merely adjoin) at the base of the posterior iridial surface.

Occasionally, in the human, a process arises directly from the posterior surface of the peripheral iris. Such an occasional finding is considered vestigial (B. S. Fine, personal communication). The entire iris, including the ciliary processes, is highly vascular. Observation of the posterior surface of the normal albino rabbit iris, following removal of the lens from the enucleated eye, shows a high degree of vascularity in the fin-shaped ciliary processes. Reports by others of

microscopic examination of sections of rabbit irides indicated that there are numerous arteriovenous anast moses (Ruskell, 1964). Although the iris is considered to function primarily as a diaphragm, the presence of these shunts suggests that the iris may also be concerned with heat regulation. Similar arteriovenous shunts, probably concerned with heat regulation, are found in areas of the skin (Bloom and Fawcett, 1968).

According to Prince and Eglitis (1964b), the lens is suspended by a ring of suspensory ligaments (the zonule of Zinn) connected to the poorly developed ciliary body. These fine filamentous structures are inserted into the anterior and posterior equatorial regions of the lens. The rabbit lens is a bi-convex structure surrounded by a partly collagenous structure, the lens capsule. The lens capsule is said to be a basement membrane secreted by the underlying lens cells. Subjacent to the entire anterior lens capsule is a single layer of cuboidal epithelial cells. Upon reaching the lens equator (Bow region) the peripheral epithelial cells extend in length. These fibers (elongated cells) continue to lengthen, extending anteriorly and posteriorly; the anterior ends of the fibers insinuate themselves under the epithelial cell layer and the posterior ends move between the capsule and existing fibers. Fibers interdigitate at the sutural regions. The rabbit lens has a visible vertical anterior and a horizontal posterior sutural region which delineates both anterior and posterior surfaces into semi-circles. The flat hexagonal fiber dimensions are reportedly 10 u by 3 u. Each fiber is bounded by a membrane approximately 6 mu thick.

Davson (1969) lists two general theories concerning the formation of posterior chamber aqueous humor. The first proposes that the plasma is filtered through the capillaries of the ciliary processes. Certain constituents of this filtrate are absorbed by the cells of the ciliary processes, then are secreted into the posterior chamber. Thereby, the aqueous humor and plasma concentrations of some substances would differ. The second theory states that the capillary filtrate may flow between the cells of the ciliary epithelium and pass into the posterior chamber. During this passage, the concentration of certain substances might be either reduced or increased by the epithelial cells.

The capillary filtration mechanism acts as follows: Large molecular weight substances (e.g., plasma proteins) traverse the capillary membrane poorly; small non-colloidal plasma constituents (e.g., creatinine, urea) cross the membrane with little hindrance. Lipid-soluble compounds (e.g., ethanol) readily pass from the capillaries.

The epithelial cells of the ciliary processes are not penetrated by the plasma proteins. Therefore, this layer of cells acts as a second impediment to the passage of these substances from the plasma to the aqueous humor. The concentration of plasma proteins in the rabbit aqueous humor compared with the plasma concentration is 50 mg% compared to 6,000 to 7,000 mg%. The epithelial cells may also serve as an impediment to smaller, water-soluble substances, such as urea. This action may be analogous to molecular sieving of column chromatography. The concentration of urea in the rabbit aqueous humor is 7mM compared with the plasma concentration of 9.1 mM.

Lipid-soluble substances readily traverse the epithelial cells and enter the posterior chamber. The aqueous humor concentration of ethanol can equal that of the plasma.

Certain substances are actively transported by the epithelial cells and, as a result, their concentration in the aqueous humor exceeds that of the plasma. For example, in the rabbit eye the relative concentration of ascorbic acid in the posterior chamber is 1.3 mM compared with a plasma concentration of 0.02 mM.

A proportion of the solutes in the aqueous humor is derived from iridial diffusion. Experiments with intravenously injected ^{24}Na indicated that the turnover of this substance is dependent on the rate of turnover of the aqueous humor as a whole. Activity of the ciliary epithelium results in a higher sodium concentration in the posterior than in the anterior chamber. There is said to be a considerable amount of diffusion of this substance directly from the iris into the anterior chamber, however. If the total rate of flow were halved (e.g., from the action of drugs) the rate of turnover in the posterior chamber is therefore said to be one-quarter the original. Such a decreased flow from the posterior chamber would result in an increased volume of fluid diffusing into the anterior chamber from the iris.

Under normal circumstances, the greater percentage of aqueous humor flows from the posterior chamber into the anterior chamber. The greater percentage of this fluid exits from the rabbit eye by percolating through the pectinate ligaments and trabecular meshwork in the angle of the anterior chamber. The humor then enters the general circulation by means

of the trabecular veins, a structure analogous to the canal of Schlemm in the human eye. In the monkey and rabbit, a small percentage of the aqueous humor (3 and 20%, respectively) exits the eye across the uveal tissue into the suprachoroidal space. Also, there is said to be a meridional flow of aqueous humor in the rabbit eye which passes through the posterior chamber and crosses the retina into the choroid layer.

Thermal currents exist in the anterior chamber of the living mammalian eye. Adler (1965) states that the currents initially result from an ascent of a portion of the aqueous humor heated by contact with the iris; upon contact with the cooler cornea the temperature of the liquid is decreased. On cooling, the density of the aqueous humor increases, causing it to descend to the inferior region of the anterior chamber. This situation assumes that the optical axis of the eye is horizontal and that the ambient temperature is much less than that of the iris. In testimony to the presence of these thermal currents, Adler (1965) cites the Kruckenberg spindle, observed clinically in certain ocular diseases. This spindle-shaped or vertical linear formation consists of pigmented material or cellular debris and is observed adhering to the posterior corneal surface. The shape of the formation is assumed to result from thermal circulation in the anterior chamber. A deposit of material is observed at the base of these formations indicating a descending flow pattern. In reference to thermal currents, Davson (1969) referred to the appearance of vertical meridional streaks of fluorescein on the posterior corneal surface following intravenous injections of the dye.

When the ambient temperature of the air is approximately 25° C, a temperature gradient exists between the cornea and the iris. Under these conditions, the highly vascular surface of the iris has a temperature of approximately 34° C, whereas the temperature of the corneal surface is approximately 31.5° C externally and 32° C internally (Schwartz and Feller, 1962). Increased ambient temperatures could result in continued heating of the corneal surface with resultant increases of the temperature of the underlying aqueous humor. If the aqueous humor in contact with the posterior surface of the cornea had been heated to more than 40° C, there would be a reversed 2° C temperature gradient between aqueous humor and iridial blood temperature. This situation might establish a reverse cycle of thermal currents in which the heated aqueous humor ascends along the posterior face of the cornea.

Lens Proteins

Crystallins and other lenticular proteins (e.g., enzymes) are synthesized in the lens from appropriate amino acids obtained from the aqueous humor. According to Cole (1970), the greater part of the metabolic energy of the lens is expended in these syntheses. The amino acids are actively transported across the lens epithelium into the lens from the aqueous humor. In general, the concentration of amino acids in the aqueous humor of the rabbit is greater than that of the plasma. Amino acids are actively transported into the aqueous humor from the ciliary processes; therefore, alterations in the iridial blood flow could alter the amino acid concentrations in the aqueous humor.

The lens fibers contain two groups of proteins based on their solubilities in lens homogenates. The three soluble proteins arranged in order of decreasing solubilities are alpha, beta and gamma crystallins. The insoluble protein is termed albuminoid. Although considered as soluble proteins, the crystallins _in situ_ are present in such high concentrations that they form an ordered gel (Kuck, 1970; Spector, 1971).

According to Waley (1969) α crystallin comprises about one-third of the lens protein. Originally, α crystallin was separated by precipitation from a lens homogenate by increasing the acidity of the solution to a pH of 5.4. This crystallin was termed α_1, by Spector (1971), as he was able to precipitate a second, nearly identical crystallin (α_2) from the same homogenate of bovine lens by the addition of ethanol to a final concentration of 13 percent. Spector (ibid) reported that the sulfhydryl (-SH) content of these two subgroups was nearly identical (approximately one-SH per $2x10^{14}$ dalton subunit. The approximate molecular weight of the α crystallin macromolecule is $1x10^6$ dalton).

In an electrophoretic field of pH 8 or greater, the α crystallins migrate a greater distance toward the anode than do β or γ crystallins. The α crystallins contain very few sulfhydryl groups (one per 20,000 subunit); this fact indicates there is little potential for the creation of disulfide bridges between adjacent molecules.

Following the precipitation of α crystallin from a lens homogenate, the β and γ crystallins remain in the supernatant. The β crystallin can be precipitated if the solution is saturated with ammonium sulfate. Davson (1969) stated that the percentage of β crystallins is variable.

Also, β crystallin has been separated by gel filtration; studies with this material indicated that the molecular weight of this group ranged from 50,000 to 500,000 daltons and that this crystallin also is made up of monomeric subunits weighing approximately 20,000. β crystallins at a pH of 8-9 migrate toward the anode at a rate less than that of the α crystallin. The sulfhydryl content of β crystallin is relatively high, suggesting a possible site for the formation of disulfide bridges between adjacent similar molecules or with other sulhydryl containing substances such as GSH.

The protein remaining in the supernatant of the lens homogenate following precipitation of both α and β crystallins is γ crystallin. Waley (1969) reported that the percentage of crystallins present as γ crystallin decreases with age. γ crystallin has been separated from lens homogenate by gel filtration. Investigations on separated γ crystallins reveal that the molecular weight is approximately 20,000 and that the sulfhydryl content is relatively high. This lens protein will precipitate at temperatures less than 10° C.

Albuminoid is the insoluble portion of the lens homogenate. The composition of this material has been determined by Dische (1970). Albuminoid is said to be the product of α crystallin and the lipophilic methylpentose, fucose. The content of albuminoid in a lens homogenate increases with the age of the lens; Dische reported that the albuminoid appeared to be associated with the lens fiber membrane. Spector (1971) suggested albuminoid may consist of α crystallin and a hexose.

Biochemical Constituents of the Lens

According to Kuck (1970) and van Heyningen (1969), four organic non-protein substances, ascorbic acid, glutathione, inositol and cholesterol, are present in relatively high concentration in the normal mammalian lens (approximate concentrations in the rabbit lens: ascorbic acid, 15 mg%; glutathione, 300 mg%; inositol, 100 mg%; and cholesterol, 70 mg%). Alterations of the concentration of these substances (ascorbic acid, etc.) have been reported in senile and cataractous lenses; transmission of light is decreased in either of these lenses. Data from biochemical studies have suggested apparent correlations between concentration decrements of both ascorbic acid and glutathione and lessened transparency in senile and cataractous lenses. Kuck (1970) indicated that inositol and cholesterol never had been implicated in lens metabolism and that the concentration alterations of inositol and cholesterol in similar cataractous lenses appeared to be related to changes of membrane permeability.

Kinoshita, Merola, Dikmak and Carpenter (1966) reported that lenticular ascorbic acid, but not reduced glutathione (GSH), decreased following a cataractogenic level of microwave irradiation. This was noted prior to any visible decrease in lenticular transparency.

In contrast, Pirie, van Heyningen and Boag (1953) found that lenticular GSH levels were decreased following cataractogenic levels of X-radiation. This occurred prior to the appearance of any visible opacities. The ascorbic acid concentration apparently was not reduced in either clear or cataractous lenses.

Ascorbic Acid

Ascorbic acid (Vitamin C) is a six carbon compound which contains one double bond and a lactone ring. Ascorbic acid (AsA) is readily oxidized to diketogulonic acid. Ocular concentrations are similar to those in kidney, brain and liver, but less than that of the adrenals and hypophysis (Heath, 1962).

There is a wide difference of AsA concentration between lenticular and aqueous humor concentration among species. The concentration in the lens exceeds that in the aqueous humor for some, but not all animals. In the rabbit eye the lenticular concentration is lower than that of the aqueous humor. Data of Pirie and van Heyningen cited by Kuck (1970a) show that the lenticular concentration ranges from 6.9 to 19 mg/100 grams (mg%) and the concentration in the aqueous humor ranged from 28.6 to 40.6 mg/100 ml (mg%). The lens is said to obtain AsA from the aqueous humor; although the aqueous humor concentration is higher than that of the lens in the rabbit eye, this situation is reversed in some species. Van Heyningen (1969) believed that this latter situation suggests the presence of an active process in these lenses. AsA is actively secreted by the epithelial cells of the ciliary processes into the aqueous humor. In this process AsA is removed from the blood plasma (1 mg%), then actively secreted. This process is dependent on both a supply of energy and a normal circulation.

The following information was excerpted from a review by Heath (1962) concerning the possible role of AsA in lens metabolism. He stated that the exact role of AsA in lens metabolism is not well established and listed its involvement in the following processes: 1) the

biosynthesis and maintenance of collagen; 2) a definite lack of AsA is associated with abnormal mucopolysaccharide formation; 3) the oxidation of AsA can lead to the formation of hydrogen peroxide, which brings about the depolymerization of hyaluronic acid; 4) AsA reportedly inhibits hyaluronidase; 5) the oxidized and reduced forms of AsA and glutathione reductase and the coenzyme NADPH are associated with an electron transport in plant tissue; 6) AsA stops substrate inhibition of p-hydroxyphenylalanine; tyrosine is metabolized abnormally in the scorbutic animal. He reported that cataract formation has not been correlated with clinical or experimental scurvy. Also, he cited experiments of Bakker in which a group of lenses maintained _in vitro_ for more than 3 weeks retained their clarity; however, all traces of lenticular AsA had vanished following the initial 10 days of incubation. In contrast, he noted that completely cataractous lenses are devoid of AsA.

Glutathione

Reduced Glutathione

Glutathione (reduced glutathione; GSH) is a tripeptide which is present within the cells of the lens and other tissues. According to Kuck (1970a) the concentration of GSH within the lens is remarkably high; e.g., concentrations of 500 mg% have been reported in the rat or cow lens cortex. He cited reported concentrations of GSH in the rabbit lens which ranged from 278 to 488 mg%. GSH is readily converted to oxidized glutathione (GSSG). GSSG is present in the lens at concentrations which are said to range from 3 to 12% of the total glutathione

(GSH+GSSG). While GSH is synthesized within the lens by means of specific enzymes, the only apparent source of GSSG is the oxidation of GSH. GSH does not diffuse from the normal lens and normally none is detectable in the aqueous humor.

According to Waley (1969) and Reddy (1971), glutathione is reported to be synthesized within the lens, as well as in other tissues. This synthesis occurs in two steps: 1) glutamic acid and cysteine are joined in a linkage in the presence of γ glutamylcysteine synthetase. 2) γ glutamylcysteine is joined with glycine in an α linkage to form γ glutamylcysteinylglycine (reduced glutathione); this second linkage required the enzyme GSH synthetase.

The enzymes responsible for GSH (γ glutamylcysteinylglycine) synthesis in the lens are also responsible for the production of ophthalmic acid (γ glutamyl β aminobutyrylglycine) a structural analogue of GSH. Ophthalmic acid is a tripeptide containing glutamic acid and glycine; however, in place of cysteine, ophthalmic acid (CA) contains α amino-butyric acid; this, in effect, results in nearly identical structures except that the sulfhydryl group of GSH is replaced by a methyl group.

The rate of production of GSH is much greater than for OA; the steady-state lens concentration of GSH is much greater than that of OA (360 and 10 mg per 100 gm wet weight of lens, respectively). No reduced glutathione has been reported to be present in the aqueous humor of rabbit eyes.

The incorporation of radioactive glycine into reduced GSH in the lens was originally reported as 2.4%/hr by Kinsey and Merriam (1950). Later, Reddy, Klethi and Kinsey (1966) published a lower value (1.8%/hr) for the incorporation of radioactive glycine and glutamic acid into lens GSH during a 24-hour incubation period. They also determined the rate for the incorporation of these two amino acids into ophthalmic acid.

Although both GSH and OA are constantly synthesized, the concentration in the lens is at a steady-state. Presumably, in order to maintain equilibrium, the rate of catabolism and removal of these substances is also at a steady-state. With respect to GSH, there are no active enzymes for the usual degradation of GSH in the lens (and presumably OA). In contrast, GSH in the liver is hydrolyzed by a specific enzyme, γ glutamyl lactamase. This enzyme, however, is either not present in the mammalian lens or, if present, is relatively inactive. Glutathione is, however, readily oxidized to GSSG. The rate of oxidative conversion (oxidation from GSH is the only method by which GSSG occurs) would be greatest, presumably, in the cortex of the lens because of the combined factors of high concentration and the proximity of GSH to oxygen dissolved in adjacent aqueous humor (there is no reported pathway for the chemical synthesis of GSSG per se). Lens membranes appear to be impervious to the passage of appreciable quantities of GSH,but permit passage of quantities of GSSG. The combined removal of GSSG by passage from the lens to the aqueous humor, as well as a low level of degradation, may serve as a path of removal and degradation of glutathione from the lens to maintain steady-state conditions.

Research of Epstein and Kinoshita (1970) suggest that one of the functions of glutathione is the maintenance of normal lens membrane permeability. They reported that an increase of membrane permeability was observed when the lens glutathione concentration was decreased markedly. GSH may serve to protect lens membrane sulfhydryl groups from oxidation. Na-K activated ATPase contained in membranes possesses reactive sulfhydryl groups. These groups appear to have undergone oxidation when the GSH levels were markedly decreased. This oxidation appeared to be related to the alteration of membrane permeability.

Glutathione is a prosthetic group for glyceraldehyde 3 phosphate dehydrogenase (Embden Myerhoff glycolytic pathway). It has been suggested that GSH may act in some manner to prevent the oxidation of sulfhydryl groups of β or γ crystallin. Kinoshita (1964) believes that the large concentration of lenticular GSH may serve to protect susceptible crystallin sulfhydryl groups from oxidation. Such protection may prevent the production of disulfide bridges between adjacent molecules which would result in an increased crystallin molecular weight or produce intramolecular -S-S bridges which would lessen the quantity of hydrophilic groups, and thus lessen the solubility. Glutathione and GSH reductase in the lens apparently act to destroy hydrogen peroxide which enters the lens from the aqueous humor. This action apparently replaces that of catalase, an enzyme which is virtually absent in the lens. The role of GSH in preventing intermolecular aggregation following the production of disulfide bridges from adjacent cysteine residues of crystallins is logical (Kinoshita, 1964). But Kinoshita and Masurat

(1957) reported that it appears that most lenticular GSH is bound or masked by the crystallins and is actually quite unreactive.

In general, however, observations of decrements of ascorbic acid and glutathione with senile or experimentally induced cataracts have not resulted in any revelations regarding the functions of these two constituents of the normal lens, nor their possible involvement in cataract production. In fact, Kuck (1970c) has stated:

> In view of our ignorance of the function of glutathione in lens metabolism,the most that can be said at present is that it must be an active metabolite since its concentration decreases with age. Another important lenticular constituent whose concentration falls with age is ascorbic acid... The significance of this change is unclear but it appears to be correlated with the diminishing metabolic activity of the aging lens.

Nevertheless, each segment of information regarding glutathione and ascorbic acid alteration gained following unique methods of inducing lens damage can be added to the present accumulation of data. Hopefully, this sum of information will some day permit a greater understanding of the function of these substances in both normal and abnormal lens metabolism.

Responses of Cornea and Lens to Injury

The stroma of the cornea contains collagen fibrils. Collagen fibers are greatly affected by "high" temperatures. When these fibrils are heated in water, and the temperature is increased to about 65° C or greater, they undergo shrinkage. The fibrils can be re-extended under tension, but they will have been denatured. Higher temperatures cause collagen to "melt," forming a gel (Verzac, 1963).

Descemet's membrane is a highly resistant tissue and can persist following erosion of the greater portion of the corneal stroma. In such instances the layer may bulge out forming a Descemetocele from inner pressure (Duke-Elder, 1970).

The single layer of endothelial cells apparently do regenerate following injury, unless the injury is widespread. According to Dikstein and Maurice (1972), these cells contain an active pump necessary to maintain the cornea in a somewhat dehydrated state. The pump appears to require sodium and bicarbonate ions and oxygen. Injury to the epithelial cells also impairs their contribution to the normal dehydrating action, resulting in edema of the stroma. During edema, collagen fibrils may not swell; rather, the interstitial ground substance imbibes water. A result of this imbibition is an uneven spacing of the fibrils. According to Maurice (1969), irregular interfibrillar spacing results in increased scattering of impinging light. Increased light scattering lessens the transparency of the edematous cornea until it becomes opaque.

During certain pathological conditions or experimentally induced states the blood-aqueous humor barrier is said to be broken down (Davson, 1969). The most obvious resultant change is an increased concentration of plasma proteins in the aqueous humor. The effect is said to result from an increased permeability of the membranes which normally restrain the passage of large molecules from the plasma to the aqueous humor. This high concentration of colloidal substances causes a flare or the so-called Tyndall effect observed when the anterior chamber is illuminated with a slit lamp. Furthermore, aqueous humor from normal rabbit

eyes clots on withdrawal; whereas, aqueous humor from normal human eyes does not. Presumably, clotting results from fibrinogen in the aqueous humor which forms fibrin in injured eyes. Aqueous humor from rabbits clots rapidly on removal. The concentration of small water soluble substances in the aqueous humor may also be altered. The concentration of lipid-soluble substances may not be appreciably altered.

The lens is avascular and, according to Duke-Elder (1970), it responds to injury by becoming less transparent and/or by being resorbed. The degree to which the lens responds to damage depends on the nature and severity of the injury and the age of the eye. The injured adult lens may respond to injury by a lessening of transparency; whereas, the young lens becomes less transparent and may be resorbed. Duke-Elder also reported that resorption of the young lens is even more common when the lens and corneal surfaces have touched and remained in contact. Apposition of cornea and lens can occur following a corneal perforation, with consequent loss of the aqueous humor. Cornea-lens apposition also can occur following dislocation of the lens, as when the zonular attachments are weakened or broken.

CHAPTER I

A REVIEW OF PUBLICATIONS CONCERNING THE ACTION OF THE CO_2 LASER RADIATION ON BIOLOGICAL MATERIAL

High power coherent radiation at 10.6 u was not achieved prior to the invention of the carbon dioxide laser by Patel (1965). The reflectance and absorption of sea water at this wavelength, however, had been previously documented. Water is essentially opaque to infrared (IR) radiation of wavelength longer than 3 u. The reflectivity of radiation at 10.6 u is less than one percent and its transmissivity at 0.003 cm of sea water at 10 u is about 10 percent (this corresponds to an absorption coefficient of approximately 760 cm^{-1} (Kauth, 1965).

Tissue has a high water content (70-80%) (Guyton, 1966). Therefore, because of this high content of water, and the high absorption coefficient of water, it was apparent that the high power output of the CO_2 laser could be hazardous to biological tissue directly exposed to the beam.

Ophthalmic Studies

Fine, Klein, Litwin, Peacock, Hamar and Hansen (1966a) published the first report on the biological effects of carbon dioxide laser radiation. The output of a CO_2 laser was focused (30 watt/sq. cm) on canine eyes and skin, implanted tumors and the hair and skin of mice. The reported irradiations of the eyes caused injury to the superficial and deeper layers of the cornea. Gross epidermal injury occurred at

exposures less than one second; with longer periods of irradiation there was progressive tissue ablation with minimal bleeding.

Fine, Zimmerman and Fine (1966) reported clinical and pathological findings following carbon dioxide laser irradiation of the eyes of rabbits. A five millimeter area was irradiated for one second intervals at various power densities. A dense white corneal opacity appeared on 15 W/cm^2 irradiations; the crater edges were thickened. Perforation of the cornea occurred on irradiation at 50 W/cm^2. Anterior lens surface concavities were always noted following corneal perforations. Similar, but smaller indentations, however, were noted in lenses of eyes excised and fixed in glutaraldehyde following non-perforating irradiations of the cornea. Irradiated non-perforated corneas healed; however, the irradiated area was opaque and scarred. Their investigations found that a clear plastic faceshield offered some degree of ocular protection against this invisible radiation.

Fine, Fine, Peacock, Geeraets and Klein (1967) published an account of a more extensive clinical and histopathological investigation concerning injury and repair following CO_2 laser radiation of the eye of the rabbit. They remarked on the lenticular indentations following high power non-perforating CO_2 laser irradiation of the cornea, but they did not observe any evidence of damage to the posterior segment of the eye. They called attention to the thickening of the corneal stroma in the region of the irradiation and to the ring of iridial pigment found on the anterior of the lens capsule. The lenticular indentations always corresponded to the site of corneal injury and appeared following

irradiations of 7 to 9 watts for one second. Figure I-1 (obtained from B. S. Fine) shows a meridioral view of a normal and an indented lens following a 9 watt irradiation of the cornea for one second, subsequent enucleation and fixation in glutaraldehyde. The arrow indicates the direction of radiation. The cornea perforated and some aqueous was expelled during a one-second irradiation at 50 W/cm^2. Also, at this power density, there were adhesions of the iris to the cornea and an accumulation of exudate in the anterior chamber. Eight days after trans-scleral irradiations, the vitreous humor was slightly clouded and in one eye a staphyloma developed. Higher power perforating scleral irradiations had vitreous strands adherent to the perforated area as well as several tears in the posterior retina.

Previously unpublished data from this study obtained from Dr. B. S. Fine (Figures I-2 - I-5) show clinical and histological appearances months following a perforating and a non-perforating irradiation of the cornea. Presumably, the perforated cornea permitted direct irradiation of the underlying lens with resultant cataractous changes.

Figures I-2D and I-2S show the clinical appearance of the right and left eye, respectively. Figure I-3D shows a section of the irradiated region of the right cornea. The break in Descemet's membrane (free arrow) indicates the cornea had perforated. Examination of the left cornea did not indicate a perforation. Figure I-4 shows the appearance of the unfixed lenses by retroillumination. Only the lens from the perforated right eye (Fig. I-4D) shows definite lens changes (the black line and spots are artefacts). Histological examination of the anterior

segment of these lenses (Figure I-5) shows cataractous changes in the lens from the perforated eye in Figure I-4D. The cataractous region is deep to the capsule. (New fibers insinuate between this region and the lens epithelial zone following irradiation.) The other lens (Figure I-4S) did not show any changes. (The clefts present in either section are artefact.)

Gullberg, Hattman, Kock and Tengrath (1967) investigated the amount of CO_2 laser energy necessary to release the blink reflex which normally has a latent period of 80 milliseconds (msec.). They found the energy dose necessary to release the blink reflex versus time followed a curve, $Q = 0.18(t)^{1/2}$ in the range 0.01 to 5 seconds (Q = calories/cm^2 and t = time in seconds). They agreed with previous findings of Fine, Hansen, Peacock, Klein, Hust and Laor (1966b) that the data fitted a simple thermal model. They also concluded that heat conduction from the surface was the principal means of loss. The blink reflex corresponded to a definite increase of corneal temperature. Barely visible corneal damage was observed following irradiation at 0.3 cal/cm^2.

Fine (1966c) indicated that the threshold for injury to the cornea should be about 100 mW/cm^2. Fine, Fine, Feigen, and MacKeen (1967) reported the threshold for permanent corneal damage following continuous CO_2 laser irradiation of the rabbit eye. In this study the irradiation periods were for "infinite time," that is, exceeding 10 minutes. Power densities studied ranged from 20 mW/cm^2 to 350 mW/cm^2, the diameter of the spot size was approximately 10 mm; therefore, the greater portion of the cornea was irradiated. Histology of irradiated corneas indicated

that the threshold for permanent injury was between 100 and 200 mW/cm^2 on continuous irradiation. The authors felt that the data obtained from rabbit eye corneas could be extrapolated to human eyes.

Feigen, Fine, MacKeen and Klein (1967) reported that hair and clothing were readily ignited and serious burns could be produced in rodent and primate skin and underlying tissues on CO_2 laser irradiation. Protective materials were investigated to determine the feasibility of their use with CO_2 radiation. Plastic, fiberglass, and rubber offered adequate protection to the head on focused 20-watt laser irradiation; the slow ablation of plastic face shields which occurred served as a warning. Glass irradiated near the edge cracked more readily than when irradiated in the center; furthermore, unbroken irradiated glass was likely to contain stress patterns so that a second irradiation resulted in fracture in a short period of irradiation. The bright light emitted from focused irradiation on glass was determined to be sufficiently intense to be hazardous to the retina if the glass was near to the eye. Quartz was found to withstand cracking on irradiation and the emitted light was less intense than that from glass. Current regulations (FDA, 1972) require that all glass lenses used in corrective eyeglasses be heat-tempered or otherwise treated to reduce shattering. Such heat treatment may predispose such lenses to shattering on CO_2 irradiation. Therefore, routine wearing of plastic corrective eye lenses in the range of CO_2 laser radiation appears to afford superior eye protection to that offered by heat-tempered glass lenses. Also, lenses made of plastic are more readily available and less expensive than those made of

quartz; however, this requires further study.

Fine, Berkow and Fine (1968) reported the presence of calcium spherules in healed corneas of rabbits previously irradiated for 10 minutes at suprathreshold power densities (greater than 200 mW/cm^2). The authors reported the distribution of these calcium spherules resulted in a clinical and histopathological appearance identical with that observed in band keratopathy in the human cornea. Although some spherules were scattered throughout the corneal stroma, the majority were in a plane located immediately beneath the epithelium. In the human eye, this plane is occupied by Bowman's membrane, the zone which is most severely affected in band keratopathy (B. S. Fine, personal communication). Some of the spherules had been phagocytized by the corneal keratocytes. but the majority were located extracellularly. They stated that it was clear that the calcium deposition located initially near the epithelial basement membrane resulted from some epithelial activity. No calcium deposition was observed within the epithelial cells by the methods used.

Campbell, Rittler, Bredemeier and Wallace (1968) irradiated rabbit eyes with CO_2 laser radiation. They used a 75-watt American Optical Unit which delivered the power through an articulated arm and hand-piece. They irradiated a 1.5 mm diameter area of each cornea for one second; the pupils had not been dilated prior to irradiation. They reported that irradiations of the cornea with power ranging from one to five watts penetrated to Descemet's membrane or perforated into the anterior chamber. The iris prolapsed in one eye following a 5-watt (approximately 280 W/cm^2) irradiation. Upon histological examination of the eye 24 hours post

irradiation, they noted coagulation necrosis of the corneal tissue surrounding the perforation, and the stromal fibers had undergone a partial hyaline degeneration. The corneal endothelial cells were completely necrotic. The anterior chamber was filled with serosanguineous exudate and the iridial vessels were distended. The lens contained a small drop of liquefied cortical material 2 millimeters beneath the anterior capsule directly in the path of the laser beam. In examination of an eye following 1 watt irradiation (approximately 60 W/cm^2), the iris was adherent to the endothelial layer of the cornea under the site of irradiation. They felt this had resulted from a break in Descemet's membrane; however, they were unable to locate it on serial sectioning of the cornea. Eyes which had been perforated sealed spontaneously with reformation of the anterior chamber. The craters in these perforated corneas filled in with opaque, ill-defined corneal infiltrates in the four weeks post irradiation. Pigment dispersion was noted on the lens surface and a small localized cataract was present at the site of perforation. They found decreasing corneal damage as the incident power was decreased from one watt to a minimum of 68 mW. They determined that the threshold for injury to the cornea was 68 mW/1.5 mm diameter (3850 mW/cm^2). They averred that this value is correct to $\pm$ 5% based on slit-lamp and histological examinations. The authors apparently did not consider that heat can flow from the irradiated site to adjacent tissues. Therefore, determination of the threshold on corneal damage based on irradiation of such a small area may be in error. They further stated that lens injury occurs only on perforation in which the lens is directly irradiated.

Peppers, Vassiliadis, Dedrick, Chang, Peabody, Rose and Zweng (1969) investigated the threshold for CO_2 laser corneal irradiation for pulses shorter than the blink reflex. The diameter of the beam ranged from 2-4 mm. Their criterion for a lesion was an opacity occurring within 10 minutes after exposure. Their threshold values were 1.2 J/cm^2 in 55 msec., 0.77 J/cm^2 in 10 msec., and 0.55 J/cm^2 in 3.5 msec. The threshold lesions healed without scarring in 24 hours. They stated that corneal damage begins when the corneal temperature increased by 35° C to a final approximate value of 67° C. They did not make any temperature measurements, but reported values determined by a theoretical heat flow model. They stated that the beam dimensions did not affect the threshold values as long as the exposed area is large compared with the depth of absorption in the tissues.

Geeraets, Fine and Fine (1969) irradiated rabbit corneas with CO_2 laser radiation at power densities ranging from 9 to 20 W/cm^2 for periods of 1 or 4 seconds. On _in vivo_ examination within 6 to 8 days following irradiation, the corneas had grayish-white opacities; several had deep central craters and/or perforations, and anterior lens indentations were reportedly visible on slit lamp examination. Nine months post irradiation the greater part of thecorneal opacities had abated leaving only a small superficial haziness. On slit lamp examination of one eye at this time, the lens was clear, but showed a small central depression of the mid anterior surface. By retroillumination, a corona of fine dust-like opacities seemed to be present in the anterior and posterior cortical region. Subsequent excision of the eyes and histological examination of the cornea from this eye indicated that the cornea had been perforated.

Litwin, Fine, Klein and Fine (1969) reported on burn injury following carbon dioxide laser irradiation. They irradiated anesthetized rabbits, monkeys and dogs. Irradiations of eyes at 5 watts for one second caused severe corneal burns, which healed but left disabling corneal opacities. When a monkey's eye was irradiated at 15 watts for one second, the cornea was perforated. They reported that the rate of soft tissue penetration was about 0.001 $cm/sec/W/cm^2$. Microscopic examination of the abdominal skin of the dogs after irradiation showed healing and regeneration of the epidermis and dermis when penetration was not deep. When the irradiation injury penetrated into the dermis, there was healing by granulation and typical burn scar formation, but damaged epidermal appendages did not regenerate. The heat injury site seemed to have been well localized under the conditions of the study. Irradiation of a dog's scalp at 24 watts for 10 seconds quickly exposed the underlying bone and caused carbonization. The authors calculated that a one-kilowatt continuous carbon dioxide laser will produce a localized soft tissue burn one centimeter deep over an area of one cm^2 within one second.

Liebowitz and Peacock (1969) described corneal damage following CO_2 laser exposure. They irradiated corneas of anesthetized rabbits. The beam diameter was approximately 6 millimeters and the duration of the pulses ranged from 0.07 to 1.0 seconds. They grouped ranges of pulse duration and energy densities according to the formula $P(t)^{1/2}$ ($P = W/cm^2$ and t = seconds). They reported that their threshold value

ranged from 5.7 to 6.7 $\left[P(t)^{1/2}\right]$ and as the value increased (14.8 - 20.2 W/cm^2, 0.07 - 0.12 second) the amount of corneal damage became greater. Irradiations of values greater than 44.2 resulted in perforation of the cornea on irradiation.

Beckman, Rota, Barraco, Sugar and Gaynes (1971) used a CO_2 laser to perform ocular surgery on rabbits. The laser had an average power output of 150 watts and a pulse rate which was variable from 60 to 300 pulses per second. They performed limbectomies with the focused output. The production of these apertures into the anterior chamber was accompanied by bubbles in the aqueous humor. All the eyes irradiated in this manner had filtering blebs which persisted for six months. They also performed keratectomies by irradiating the cornea with a ring-shaped beam pattern made by focusing the output with an Axicon lens. Following a partial penetrating keratectomy, the rim of the ablated area appeared white without evidence of charring. The edge of the keratectomy was not as sharp as if the operation had been performed mechanically. The corneal button formed on penetrating keratectomy was pyramidal in shape. They remarked that the pyramidal appearance of the button resulted from the fact that cutting occurred more rapidly in the anterior cornea than in the posterior layers because of the divergence of the beam exiting tha Axicon lens. They stated that one should be able to use CO_2 radiation to remove cylindrical or mushroom-shaped corneal grafts if a circular beam could be obtained with less divergence. The authors apparently neglected to consider that the pyramidal shape of the corneal button may have been due partially to heat-induced shrinkage of stromal collagen

fibers. During irradiation, the outermost stromal layers may have been raised to a higher temperature than the subjacent layers near the aqueous humor.

Dental Investigations

Lobene, Bhussary and Fine (1968) investigated the effect of focused CO_2 laser radiation on the enamel and dentin of extracted human teeth. They focused the output of a 20-watt continuous wave (c.w.) CO_2 laser to 1-4 mm spots by means of an Irtran II (zinc sulfide) lens. The resultant power densities ranged from 150 to 2,400 W/cm^2; exposure times ranged from 0.1 to 5.0 seconds. They reported that enamel in irradiated areas was fused, chalky and opaque. Immediately post irradiation the irradiated areas, including the crater, were hot to the touch. Attempts to fuse hydroxyapatite to enamel failed. X-ray diffraction patterns of irradiated enamel did not show any alteration of the normal hydroxyapatite pattern, but did show evidence of alpha calcium orthophosphate. *In vitro* heat transformation of hydroxyapatite to alpha calcium orthophosphate occurs when a temperature of 1,400 C is attained. Histological examination of the irradiated material showed that cracks extended through the enamel and penetrated the dentin. The rod substance of the enamel had been disrupted and the inter-rod substance had been incinerated. It appeared that incineration of the orthodontic process had occurred.

Scheinen and Kantola (1968) investigated the effect of CO_2 laser irradiation focused (300 W/cm^2) for 1 to 3 seconds on extracted human

teeth. These irradiations resulted in craters whose depth was a function of the irradiation time. Microscopic examination showed that the temperatures reached were sufficient to melt and vaporize the enamel hydroxyapatite. Part of this material solidified and then condensed at the periphery of the crater resulting in an elevated rim. The organic constituents were incompletely combusted at the periphery of these craters.

Scheinen and Kantola (1969) carried out microradiography and polarized light microscopy on the craters resulting from focused CO_2 laser irradiation of the buccal surfaces of extracted teeth. They reported extensive cracking of the enamel in the crater region; this cracking extended to the dentin. The outer layers of the lasered enamel were highly radio-opaque; this was felt to have resulted from plume condensation and recrystallization. The radiolucency of the material immediately beneath this region was thought to have indicated an area in which a large proportion of the hydroxyapatite had been vaporized or melted. The polarized light microscopy of areas adjacent to the crater indicated regions of reduced amounts of ordinarily negative birefringent crystals. The peripheral dentine was remarkably X-ray absorbent indicating a high degree of mineralization probably associated with the vaporized or melted enamel.

Stern (1970) compared the demineralization of "artificial caries" produced in CO_2 laser irradiated and control spots of dental enamel. He irradiated the buccal side of human third molars with approximately 160 W/cm^2 for 0.1 or 0.5 seconds in a 7-mm diameter spot size. The

control and irradiated sections were obtained from the same non-carious teeth which had been previously frozen immediately following extraction, then thawed and sectioned. The entire section of tooth was covered by wax except for the 7-mm diameter irradiated or control region. The waxed sections were immersed in lactic acid and hydroxymethylcellulose solution (pH 4.5) at 37.5° C for periods ranging from one to two weeks. Each section was removed at the first sign of a white "carious" spot. Sections made through these regions were X-rayed. He reported that the 0.1 second CO_2 laser exposure reduced the surface demineralization "caries." Although he felt that there might be damage to the pulp, the use of higher power would permit shorter pulses and thereby reduce the heating to the material subjacent to the enamel.

Weichman and Johnson (1971) reported on their unsuccessful attempt to seal the orifice of root canals of teeth in vitro with the focused output of a CO_2 laser. The laser output was variable from 5 to 200 watts, the spot size from 0.005 to 0.1 inch in diameter and the pulse duration from 0.1 msec to 3 seconds. One unit employed was capable of emitting single to multiple pulses (100 p.p.s.). During irradiation the target area was cooled by a stream of high velocity argon gas. They reported that the lasered site became brown at the threshold for damage; with increasing power and pulse duration, charring occurred followed by melting of the cementum and dentine and eventually a porcelainized surface formation occurred. The porcelainized surface formation unfortunately was not fused to the underlying material. This lack of fusion indicated to the authors the apparent unfeasibility of this technique for sealing

off root canal orifices. They felt, however, that the method had possibilities and stated they would attempt to create a fused region by the addition of powdered dentin to the target area prior to laser exposure.

Surgical and Medical Applications

Fine, Hansen, Peacock, Klein, Hust, and Laor (1966b) reported biophysical studies with the CO_2 laser. They discussed a heat flow analysis of threshold lesions in rodent skin and tissue ablation in rabbit skin produced by CO_2 laser radiation. They reported that the absorption coefficient for tissue at 10.6 u ranged between 130 cm^{-1} and 270 cm^{-1}; therefore, radiation at this wavelength is attenuated by 64 percent at depths ranging from 37 u to 77 u, respectively. They carried out calculations based on both an opaque and a diathermatous tissue model when performing their heat-flow analysis. The temperature elevation at the skin surface obtained by thermocouple measurements of skin on CO_2 irradiation were in agreement with their predicted values.

Yahr and Strully (1966) reported a method for blood vessel anastomosis which utilized the focused output of the CO_2 laser. In their method, one side of the donor vessel near the distal wall was glued to a wall of the recipient vessel with Eastman 910 monomer (2-methyl-cyanoacrylate). Then an aperture was created in this common wall of the two vessels with the focused output of the CO_2 laser; the open end of the donor vessel was then closed creating a side-to-side anastomosis. They reported that care had to be taken during exposure in order that a second aperture was

not created in the opposite wall of the recipient vessel. They also reported that the focused CO_2 laser beam permitted clean, relatively blood-free incisions of skin, liver, kidneys, lung and bone. They did not present data, however, to substantiate this statement. Based on our own unpublished results, this appears to be unlikely.

Mullins, Jennings and McClusky (1968) utilized the focused output of a 50-watt CO_2 laser in studies on the reaction of portions of rhesus monkey liver. Histology was done on the irradiated area and adjacent tissue. Also, serum enzymes were monitored as an index of liver damage.

They were able to cut and seal vessels with diameters up to 3 mm; however, it was necessary to constantly remove blood flowing from larger cut vessels because it absorbed the radiation and halted cutting.

The concentration of many hepatic enzymes was increased in the circulating blood during the 24 hours following surgery; the levels of nearly all returned to normal within the ensuing two weeks.

Histological examination done one or three months after liver resection showed adjacent areas of complete cellular destruction with prominent vacuolization and necrotic cells. Their summary of CO_2 laser surgery of the liver was that it was bloodless, allowed fast dissection and there was no sign of continuing organ injury, and the healing was uncomplicated. This conclusion is probably not justified—they had problems in cutting larger vessels.

Brownell, Parr and Hysell (1969) determined the threshold values for CO_2 laser damage to the skin. They irradiated the unpigmented areas of Yorkshire pigs because they considered that this tissue most closely

resembled human skin histologically. The diameter of the irradiating beam was 0.75 inch and the power densities ranged from 0.69 to 13.6 W/cm^2. They obtained injury reactions at both threshold and suprathreshold levels. They defined a threshold lesion as one in which the erythema disappears witin 18 to 24 hours. Their lesions ranged from threshold to white, coagulated burns. The burns were evaluated 18 to 24 hours after laser exposure and the analyzed data reported as the median Effective Exposure Time (EET_{50}), i.e., the exposure time for a given irradiance (with a 50% probability) of producing a given grade of lesion. The EET_{50} ranged from 0.22 second at 13.6 W/cm^2 to 21.6 seconds at 0.69 W/cm^2 for mild erythema; 0.43 seconds at 13.6 W/cm^2 to 33.1 seconds at 0.69 W/cm^2 for moderate erythema; 0.54 seconds at 13.6 W/cm^2 to 46.7 seconds at 0.69 W/cm^2 for severe erythema, and 0.66 seconds at 13.6 W/cm^2 to 39.6 seconds at 0.74 W/cm^2 for a white spotty burn.

Fox (1969) reported on his investigation of the use of focused CO_2 laser output as a surgical "light knife." This study employed a specifically constructed apparatus in which 30 to 35 watts were delivered through an articulated arm. The output beam was 2 millimeters in diameter.

He reported that nearly blood-free incisions of tissue could be produced except when blood vessels larger than 1 mm diameter were cut. When vessels of this size were cut the resulting hemorrhage absorbed the laser energy and halted further cutting. Similar problems were encountered when he attempted to incise brain or liver. Only the surface

of bone could be readily cut. Although glasses or plastic goggles were worn by the surgeon and his assistants for protection, the invisibility of the beam caused accidental burns either by direct irradiation or from reflection of the beam from metal surgical equipment.

Ketcham, Hoye and Riggle (1970) mentioned use of the CO_2 laser to perform a partial hepatectomy and also reported that the bleeding during such an operation was minimal unless a major vessel was involved.

Leon Goldman (1970) stated that the use of the invisible CO_2 laser in biomedical applications required the simultaneous use of a Helium-Neon laser to locate the beam. He furthermore reported that quartz glasses are now being used with high power CO_2 units.

Wilkening (1970) stated that the threshold data for the CO_2 laser was nearly absent according to his literature survey. (The threshold data of Fine, Fine, Feigen and MacKeen [1967] had been previously reported.) He did remark that Zweng had presented "bench-mark" data values at the 33rd annual meeting of the Industrial Hygiene Foundation. He further remarked on the complete absence of threshold data for UV lasers. He stated the necessity for appropriate protective eye wear for use with each laser.

Rockwell (1970) discussed the application of the CO_2 laser output in tissue cutting and reported investigations carried out to determine the conditions required. He stated that the power densities required ranged from 10^3 to 10^4 W/cm^2. He measured the power density and cutting speeds required to incise rat abdominal skin. He accomplished this by passing the anesthetized animals under the focused beam (0.3 to 0.6 mm

diameter), thus irradiating the skin surface at controlled rates. He reported power required ranged from 1.25 watts and cutting speeds varied from one to 20 mm/sec.

Goodale, Okada, Gonzales, Borner, Edlich and Wangensteen (1970) utilized the output of a CO_2 laser to halt bleeding from experimentally produced gastric lesions. The lesions were produced in dogs undergoing a constant infusion of heparin and histamine. The lesions were produced as follows: the stomach was exposed by a laparotomy and a deep ulcer created in the internal gastric wall with either a pumped jet of HCl or by abrasion with steel wool. At this time a gastrostomy was performed and the blood loss determined by weight following the sequential application of tared paper discs to the lesion. Then the CO_2 laser gastroscope was introduced orally into the stomach; the bleeding site was irradiated (12 W/cm^2 over a 3 cm^2 area) for 3 to 5 seconds continuously. Then the blood loss was determined again. The experiments were repeated using a monopolar ball electrocautery unit in place of the laser. Hemostasis was accomplished 60 times faster with the laser than with the electrocautery apparatus; furthermore, there was a significant decrease in the amount of blood lost from the lesions following laser irradiation over that lost from the electrocautery-treated lesions. There did not appear to be any observable histological differences between the areas irradiated with the laser and those treated with electrocautery. The authors felt that this CO_2 laser method of treating hemorrhaging stress ulcers would be preferable to operative measures in the moribund patient.

Gonzalez, Edlich, Bredemeier, Polanyi, Goodale and Wangensteen

(1970) compared the efficacy of the CO_2 laser output with an electrocautery apparatus in the management of liver hemorrhage in dogs. They used a device wherein the output from a CO_2 laser was directed through an articulating arm to a hand piece. The output power density was 12 W/cm^2 (45 W); the electrocautery was a monopolar ball tip electrocoagulation needle. Standard subcapsular lesions (8 x 9 x 0.5 cm) were created sequentially on separate lobes of the same liver. One group of animals was pretreated with sufficient heparin to delay clotting time threefold. The other group was not pretreated. Hemostasis of one lesion was accomplished with the electrocautery needle, then the second lesion was created and hemostasis was accomplished with CO_2 laser output. The hemostatic ability of the laser output was significantly greater than that of the electrocautery. Also, the electrocautery adhered to the tissue and required the constant removal of denatured blood.

The incisions were closed and the animals were kept for observation and subsequent histologic examination. Histology showed that the zone of adjacent tissue necrosis in laser-treated livers was five millimeters deep.

Polanyi, Bredemeier and Davis (1970) discussed the use of an American Optical Company (AO) laser for surgical procedures. The laser unit delivers a useful output of 50 watts. A shutter arrangement allows pulse lengths from 0.1 to 6 seconds; this may be positioned for continuous use. Visualization through the associated endoscope attachment of this unit is permitted by means of a time-sharing arrangement. The line of sight illuminating light and the laser beam can be directed down a common path to the target site.

They reported that most tissues except bone are cleanly cut by burning or vaporization when the beam was focused to a diameter of 1 millimeter or less. The output has been used for the removal of cancerous tissue. The laser output has been used experimentally for the destruction of parietal cells of the gastric mucosa in cases of gastric ulcer. They reported that the output had been used to produce experimental cardiac myopathies, as well as partial and complete heart block; others had utilized the laser to create lesions in the brain and spiral cord.

Stellar, Polyani, and Bredemeier (1970) reported the results using the AO CO_2 laser in cutting a variety of tissue; dogs and cats were used as experimental animals. They attempted to determine the optimal conditions by varying the intensity, the diameter of the irradiating beam, or the rate at which the cutting portion of the beam swept over the tissue. They found the laser beam cut all soft tissue readily and hemostasis was excellent. At a power of 40 watts or less, bone charred and ignited. Skin, muscle and fascia were cut in one "nearly bloodless sweep" of the laser beam. Underlying tissue was protected from the beam by covering with a wet section of gauze.

Investigations were carried out on neoplasms transplanted in mice. In order to laser these tumors, the overlying skin was reflected, then the tumor was vaporized until its base was reached. Finally, the base of the tumor was vaporized, making an effort to destroy all neoplastic tissue visible under the operating microscope.

Lesions were produced in the brain and spinal cord of cats with the focused laser output (a few watts 0.3-0.5 sec. exposure). This operation was best accomplished through the dura following craniectomy or laminectomy; this technique produced a sealed lesion without spinal fluid leakage. In cases in which boiling of cerebrospinal fluid from the cord dissipated a portion of the laser energy, a second exposure was required to create a suitable lesion. Animals were killed for histological examination at one hour, at four and 14 days, and at one and three-month periods. The walls of the hole in brain tissue were smooth and the depth of the surrounding damaged tissues was less than a fraction of a millimeter.

They suggested that once exposed, large areas of the brain, e.g., gray nucleus, may be vaporized away; similarly, spinal tracts involved in pain transmission might be destroyed with the CO_2 laser output.

They reported some apparently anomalous results following tumor removal experiments. Some tumors recurred when all the neoplastic material had apparently been removed. Conversely, tumors did not recur in some experiments in which neoplastic material had been deliberately left. They plan to determine the extent of certain tumors by monitoring the radioactivity of the smoke produced during CO_2 laser removal. Certain tumors will take up a considerable amount of I^{131} or P^{32} labeled albumin injected intravenously immediately prior to the operation. (One must consider, however, that tumors may not selectively take up radioactive compounds.)

They felt that the CO_2 laser treatment of decubitus ulcers ("bed sores") could readily clean and denude an area of dead tissue.

Disadvantages include the invisibility of the beam, also the danger of fire or explosion caused by interaction of the beam and flammable material and gases. The author concluded that reading spectacles provided adequate protection from energy inadvertently reflected from metal surgical instruments. (See note included in this chapter by Feigen, Fine, MacKeen and Klein [1967].)

Campbell and Fine (1970) reported thresholds for heat sensation on irradiation of human skin <u>in vivo</u> with the output of a CO_2 laser. They irradiated 3, 7, and 12.5 cm^2 areas of the dorsal area of the hand of two Caucasian males. Their data was compared with that of Brownell, Parr and Hysell (1968) reporting the power density, area, and exposure duration required to produce erythema on depilated porcine skin.

Campbell and Fine

RT (seconds)	Watts/cm^2	Area(cm^2)
1	0.320	3
0.75	0.700	3
15	0.290	7
0.75	0.700	7
30	0.195	12.5
0.75	0.700	12.5

RT (reaction time) time elapsed between exposure onset and response to the sensation of warmth.

Brownell et al. (1968)

T (seconds)	Watts/cm^2	Area (cm^2)	
21.6	0.69	3.4	Mild erythema
0.37	7.6	3.4	
39.6	0.74	3.4	White spotty burn
1.1	7.4	3.4	

T, exposure time

Goldman and Cowards (1970) reported the effects of irradiating metastatic human melanoma with a 25-watt CO_2 laser output. They found that non-specific thermal coagulating necrosis developed with no local dissemination of tissue fragments. (In our studies, we found that the heat generated on irradiation produced pain. The authors did not discuss their methods for overcoming this problem.) They reported the production of partial or complete heart blockage in dogs on 30-watt irradiation with an AO CO_2 laser unit. They furthermore mentioned their work on liver surgery where the heat transmission, histochemistry and liver function data will be reported later. They controlled bleeding from large vessels with gel foam packs and thrombin and also experimented with methods for welding liver tissue. They reported one member of the group had used the CO_2 laser output in the control of experimentally produced gastric hemorrhage in dogs. This short report further discussed the use of 80-watt pulsed high frequency CO_2 laser; the

device cut tissue well but produced hemorrhage and plumes of blood. They reported that they had removed a human epithelioma from the neck of a patient with the CO_2 laser because of the great deal of associated vascularity; they experienced a minimal amount of associated bleeding. They removed a digital angiosarcoma with a scalpel, a CO_2 laser beam and a high frequency electrosurgical unit and reported that based on the relative merits of each technique, the CO_2 laser appeared to have been best. Because of the small amount of data reported in any one area of investigation, their conclusions should be viewed as tentative.

CHAPTER II

SIMULTANEOUS SURFACE AND ANTERIOR CHAMBER TEMPERATURE MEASUREMENTS DURING CARBON DIOXIDE LASER IRRADIATION OF THE CORNEA

Fine, Hansen, Peacock, Klein, Hust and Laor (1966b) reported that the absorption coefficient, α, of tissue at 10.6 u ranged from 130 cm^{-1} to 270 cm^{-1} and that its reflectivity was less than 1% at that wavelength. Furthermore, it is probable that the scattering in tissues is not significant at that wavelength. Consequently, at low irradiation power levels where there is no significant tissue alteration, 64% of the incident electromagnetic energy (I_o) would be absorbed in the outermost 78 to 37 u of tissue, respectively; and an equal percentage of the residual transmitted energy absorbed by the underlying 78 to 37 u of tissue and so on. Under these circumstances, where there is no significant alteration in the absorption coefficient, α , with time, the transmissivity of the corneal tissue will obey an essentially exponential relationship:

$$I = I_o e^{-\alpha x},$$

where I is the radiation intensity at a depth from its front surface within the corneal tissue, I_o is the incident intensity, α is the absorption coefficient, and x is the depth within the cornea.

Based on this exponential relationship and utilizing the lower of the two absorption values quoted above, the maximum percentage which could be transmitted through a 500 u thick adult rabbit cornea would be 0.0015%. As a result, essentially all the radiation at 10.6 u would be

absorbed within the cornea under the above conditions. Even if the radiation is essentially absorbed within the cornea, however, there will be heating of the underlying aqueous, iris and lens on CO_2 irradiation of the eye.

Fine, Fine, Feigen and MacKeen (1968) also reported that the threshold for corneal injury on continuous CO_2 irradiation of the cornea was 100- to 200 mW/cm^2.

The purpose of this chapter is to determine the corneal surface steady-state temperatures as a function of power density under CO_2 irradiation, including the temperatures on irradiation at threshold levels for injury. A second purpose of this study is to determine the aqueous humor temperature under these irradiation conditions and the relationship between the corneal surface and anterior chamber temperatures. From these data it may be possible to estimate the steady-state aqueous humor temperature elevations during CO_2 irradiations of the cornea by measuring the corneal surface temperature.

Materials and Methods

The CO_2 laser used in this investigation is described in Appendix 1 and shown in Figure A-1-1. The beam was expanded by means of a concave front-surface mirror. A homogeneous portion of this beam was selected by means of its pattern produced on Thermofax paper. A beam selector with an exit aperture of 1.3 cm^2, shown in Figure II-1, was placed in this portion of the radiation. Varying the distance between the beam selector and mirror varied the power density at the beam

selector exit port. Power densities below 250 mW/cm^2 were measured with a calibrated Eppley thermopile (Eppley Corp., Newport, R. I.). Greater power densities were measured with a Coherent Radiation Laboratory Model 201 detector. A piece of Thermofax paper was temporarily affixed to the exit port prior to animal irradiation. Essentially uniform darkening of the 1.3 cm^2 diameter area indicated that the entire exit port area was irradiated.

Fifty adult male and female albino rabbits were used in this study. Each was systemically anesthetized with I.M. injection of Innovar Vet (fentanyl 0.4 mg/ml and droperidol 20 mg/ml) plus atropine sulfate to control respiratory distress. The eyes were topically anesthetized with Ophthetic drops (proparacaine HCl, Allergen) dilated with Mydriacil solution (bis tropicamide 0.5%, Alcon), and held open with a metal speculum (lid retractor). The eye of the rabbit was positioned behind the exit port so that its corneal surface was at the approximate level previously occupied by the heat sensing disc of either power detector. Visual inspection through the beam selector assured complete irradiation of the corneal surface in the speculum-opened eye.

Surface temperature determinations were made with a flat-ended chromel constantan thermocouple (Baldwin Lima Hamilton No. TC-RC-FS-100). Anterior chamber temperature measurements were made with a smooth-ended sheathed thermocouple made of the same metals (BLH No. TC-RC-IS-100). Reference junctions (Fig. II-2) made of the same metals were secured in a continuously stirred, constant temperature water bath maintained at 33° C (a Gilson Warburg Bath containing approximately 60 liters

of water). The room temperature was maintained at 20-25° C by means of an air conditioner. The thermocouple outputs were amplified by separate Hewlett Packard 412 Vacuum Tube Voltmeters and were recorded on separate channels of a Massa Cohu PR 201 Recorder.

Once the anesthetized animal was positioned at the exit port of the beam selector, both thermocouples were calibrated at the temperature of the reference junction (33° C) and at a higher temperature (e.g., 45° C) in a Dewar flask filled with water monitored with a mercury thermometer. An opening was made into the anterior chamber of the eye immediately anterior to the limbus with a 26-gauge hypodermic needle. The smooth-ended thermocouple was inserted via this opening taking care to avoid touching the iris or lens surface. Unless otherwise noted, the heat-sensing tip was positioned approximately in midchamber along the eye's axis. The anterior chamber temperature was allowed to equilibrate for several minutes. The flat-surface thermocouple was held lightly on the corneal surface until a constant reading was obtained. At this point the irradiation was begun. The surface thermocouple was removed after a steady-state temperature was reached and replaced for periodic monitoring. It was replaced prior to cessation of radiation and held in place until the corneal temperature fell to, or approximated, pre-irradiation levels. Occasionally, after the surface and anterior chamber temperature had plateaued, several drops of saline were applied to the cornea to determine its effect. This was done primarily between irradiation power densities of 200 to 600 mW/cm^2.

Because of possible beam inhomogeneities (i.e., variations of the beam), the external thermocouple was moved gently over the surface of the cornea during the irradiation in order that temperature differences might be measured.

In two instances, it was possible to move the sensing tip of the inserted thermocouple in various parts of the anterior chamber during the irradiation. In these situations, the temperature was measured in various regions of the anterior chamber and in the region posterior to the iris during the irradiation.

Studies of the radiation absorption by the isolated thermocouple, dry and wet, were carried out in air at power densities up to 1,000 mW/cm^2; the reference thermocouple was immersed in a Dewar flask filled with water at 20° C. The thermocouple was moved vertically and laterally in the beam approximately one centimeter in front of the power detector opening at each power density level.

Results

Gross Appearance of the Anterior Portion of the Irradiated Eye

No visible alterations of the corneal surfaces were observed during the exposures (approximately 10 minutes) when the power density was 100 mW/cm^2 or less. Pitting of the corneal surface was observed, however, during higher power density irradiations (200 mW/cm^2 or greater). The extent of the pitting increased with increased power densities, as reported previously (Fine, Fine, Feigen and MacKeen, 1968). In addition to the pitting of the cornea, exposures greater than 380 mW/cm^2

caused corneal opacification. The rapidity of appearance, as well as the degree of opacification, appeared roughly a function of the power density; that is, at 380 mW/cm^2, the opacity produced was slight and appeared after eight minutes of exposure; whereas, at 780 mW/cm^2, the opacity produced was pronounced and appeared after only about three minutes of irradiation.

A third type of corneal alteration was noted during these higher power density irradiations (greater than 380 mW/cm^2). The investigator moving the hand-held thermocouple over the corneal surface sensed the gradual appearance of "hard spots." The majority of these "hard spots" were in the superior portion of the cornea, although they were also sensed in the mid portion of the corneal surface.

Prior to irradiation, the pupillary diameters were approximately 10 mm following the administration of the mydriatic. Following power density irradiations greater than 500 mW/cm^2 the pupils appeared constricted; the pupillary diameter was reduced to appro 'mately 5 mm.

In Vivo Temperature Measurements

Although the length of time varied during which the eyes were held open with the metal speculum prior to irradiation and the room temperature was not maintained absolutely constant (20-25° C), our pre-irradiation temperatures (cornea, 32.3° C, anterior chamber, 34.4° C) are in general agreement with those obtained under carefully controlled conditions by Schwartz and Feller (1962), Table II-1.

When presure was exerted by the thermocouple pre-irradiation which was sufficient to indent the anterior corneal surface, there was always a concomitant temperature rise measured by the thermocouple. In unirradiated eyes, this temperature elevation as recorded by the thermocouple was less than 0.5° C for the slight indentations caused during a recording, and it could be increased to as much as 1.8° C for intentionally caused maximum indentation of the cornea. Similar temperature elevations on increased corneal pressure were also found during irradiation. These temperature elevations could be easily detected on the chart recording and were minimized by a corresponding decrease in the thermocouple pressure on the corneal surface.

Upon initiation of irradiation, the temperature on the corneal surface and in the anterior chamber rose in 3 to 5 minutes to a steady-state level. The corresponding temperature elevations at the corneal surface and in the aqueous humor at different power densities of laser radiation are shown in Table II-2. These elevations are plotted in Figure II-3 as a function of incident corneal power density. The aqueous humor temperature elevation was always less than, and lagged behind, that of the anterior surface of the cornea. Upon cessation of irradiation, both temperatures fell slowly toward pre-irradiation levels.

The temperature of both the corneal surface and the aqueous humor decreased approximately one degree on instillation of the normal saline during irradiation. The temperature returned to that observed prior to instillation within several seconds.

The average aqueous temperature elevation is plotted versus the average corneal temperature elevation in Figure II-4. Initially, the relationship between the aqueous and corneal temperature elevation is linear. In the central part of the curve, the rate of elevation of aqueous humor temperature relative to the rate of corneal temperature elevation (the slope of the curve) appears to be less than initially. At a power density level of 770 mW/cm^2 the rate of aqueous temperature elevation appears to again be high relative to corneal temperature elevation in comparison to the central part of the curve.

At a room temperature of 20° C, the pre-irradiation temperature beneath the iris of two eyes averaged 0.7° C higher than that at the exposed lens surface. During corneal irradiation two determinations were made of temperature in different regions of the anterior chamber and behind the iris. The temperatures are tabulated in Table II-2. At these power densities, the anterior chamber temperature decreased as the lens surface was approached, and the lens surface temperature beneath the iris was less than either the anterior iris surface or exposed lens surface temperatures.

Shielding of the corneal surface thermocouple tip from the irradiation with a 2-mm strip of aluminum resulted in a corneal surface temperature which was minutely less than the steady-state temperature previously recorded with the unshielded tip. With continued application of the shielded thermocouple the corneal temperature slowly decreased a slight amount from the steady-state temperature. There was also a delayed decrease in the recorded temperature from the anterior chamber.

Thermocouple Irradiation Measurements

Because of the high reflectivity of metals to radiation at 10.6μ and the high absorptivity of water at this wavelength, direct irradiation of the thermocouple, dry and wet in air was carried out. The results are tabulated in Table II-4. The temperatures of the isolated thermocouples rose to a peak level within less than 1-1/2 seconds. The temperature increase of the dry thermocouples was always greater than that of the wet. Irregularities of the thermocouple output on movement in front of the power meter aperture were no doubt the result of variations within the beam. (The time response of the isolated thermocouple was qualitatively evaluated by moving it in and out of the beam.)

Discussion

Occasionally, corneas with surface irregularities are observed in unirradiated rabbits; such animals were not used in this experiment. Furthermore, the pitting did not appear to have resulted from drying. Some corneas were moistened during irradiation; also, no pitting was observed in corneas irradiated at less than 100 mW/cm^2.

Pitting of the corneal surface noted following protracted low power irradiations (200-300 mW/cm^2, 10 minutes or longer) could have resulted from either uneven corneal irradiation caused by beam inhomogeneities, a difference in the response of the individual corneal cells to the radiation, or differences in response of the intercellular regions. It does not seem that beam irregularities alone could have caused the corneal pitting (cratering) during this relatively long exposures because

movement of any "hot spots" within the beam, thermal diffusion within the tissue, plus slight movements of the eyes would have prevented continuous intense irradiation of specific small areas.

Carbon dioxide laser radiation at 10.6 u is strongly absorbed by water (Fine, Hansen, Peacock, Klein, Hust and Laor, 1966b). Therefore, corneal pitting on CO_2 laser irradiation may have occurred in the following manner: Because of its high water content, the tear film was probably evaporated initially. Let us assume that corneal epithelial cells have an equal concentration of water, and that the major absorption on irradiation is by this water in the cell. The water content of the cell is dependent on its volume. The larger the cell, possibly the greater its total water. If the cell surface offers the maximum resistance to heat flow, then the resistance to heat flow would be dependent on surface area; that is, the heat dissipation would be dependent on its surface area. The larger the cell, the greater the volume-to-surface area ratio. Therefore, some cells in any one layer may have reached a critical temperature earlier than others. Resultant ablation of these cells may have produced a non-uniformity of the surface. The cellular residue at the sides of the adjacent remaining epithelial cells might have reflected some of the CO_2 laser radiation, thus producing a non-uniform energy absorption. If some of of this reflected radiation were directed to the base of the crater, the reflected radiation plus the directed radiation would have increased the intensity of radiation at the base of the crater. The crater would have, thereby, acted as a Mendenhall wedge. This would have caused an increased temperature in the base of the crater, and a resultant deepening of the crater.

Other alternatives, involving non-uniform response of the intercellular elements to irradiation at this wavelength, or differences in the physical strength of the desomsome linkages might be of importance insofar as the pitting is concerned. The mechanism for occurrence of pitting is not understood at present; more work involving electron microscopy (transmission, or scanning) will be required to understand the pitting mechanism.

Under normal circumstances the pupillary diameter changes in response to alterations in the intensity of the light entering the eye. The resultant changes are generally considered to be mediated by parasympathetic or sympathetic pathways to the sphincter, or dilator muscles of the irides (Adler, 1965). Pupillary meiosis on the heating of the eye has been observed by Goldman (1933b) and by Hoffman and Kunz (1934).

According to Goldmann, the meiosis is caused by thermal damage to the iris; Hoffmann and Kunz (1934) did not comment on the cause of the mechanism. Prince and Eglitis (1964a) cited the work of Longworthy and Ortega that indicated that meiosis occurred in rabbit eyes when the blood pressure was elevated; the resultant engorgement of vessels was thought to cause the decreased pupillary aperture.

In this study, a dilatation of iridial vasculature was observed during and following laser irradiations. It is possible that increased blood flow associated with vascular dilatation occurred in response to heating. Thus, the meiosis observed in this study may have resulted from transient iridial vasodilatation due to temperature elevation in the anterior chamber.

A second possible cause of the meiosis may have been the direct action of heat on the iridial musculature. According to Guyton (1966), the stimulation of heated muscle results in an increased contraction (the increase is a direct function of temperature, within limits). It is possible that the temperature elevation of the sphincter muscle which is in the pupillary margin was greater than that of the opposing radially oriented iridial dilator muscles because the latter were surrounded to a greater extent and cooled by adjacent iridial vasculature. Therefore, if the nervous stimulation to the iris remained similar to that prior to irradiation, the constrictor muscle groups heated to the higher temperature would have contracted more than the relatively cooler dilator muscles. Therefore, this situation also would have contributed to the pupillary constriction observed.

In previous investigations (Fine, Fine, Feigen and MacKeen, 1968), permanent injury to the eye was not detected at power density levels of 100 mW/cm^2, whereas permanent corneal injury was observed at power density levels of 200 mW/cm^2. Average corneal temperature elevations of 5.5 and 8.7° C were recorded for power densities of 100 and 270 mW/cm^2, respectively. Although the corneal stromal temperature was not measured, indeed it may have been higher than the measured corneal surface temperature because of heat flow and surface cooling. Pitting of the corneal surface was noted, however, at irradiation levels above 200 mW/cm^2. Consequently, a steady-state corneal surface temperature elevation of considerably less than 10° C rise appears to result in permanent injury. It is therefore also possible that radiation which will result in a steady-state temperature elevation of less than 10° C in a specific ocular tissue, particularly

if it is cellular, such as the retina, may result in injury to that tissue.

At ambient temperatures of 20-25° C, the corneal surface temperature was lower than that of the aqueous humor. This relationship continued under low power CO_2 laser irradiation of the cornea until power densities of about 270 mW/cm^2 were reached. At these power densities, which are above the predetermined threshold value for permanent corneal damage, the steady-state temperature at the corneal surface was higher than that of the aqueous humor.

For power densities up to 770 mW/cm^2, the approximate steady-state temperature increase of the aqueous humor can be determined in an eye undergoing continuous CO_2 laser irradiation from recordings of corneal surface steady-state temperature (Figure II-3). At each CO_2 laser irradiation power density, the steady-state temperature increase of the cornea exceeded that of the underlying aqueous humor. The elevation of aqueous temperature, however, is not linearly related to elevations of corneal temperature (Figure 11-4). At low power densities, there appeared to be a linear relationship between the temperatures. As the power density was increased, there was a decrease in the rate of rise in aqueous temperature as a function of corneal surface temperature. At the highest power densities studied, the rate of rise of temperature in the aqueous as a function of corneal temperature again increased.

There are a number of factors which contribute to this aqueous-to-corneal-surface temperature relationship. These include: an absorption coefficient of 130-270 cm^{-1} (Fine, Hansen, Peacock, Klein, Hust and Lacr, 1966b) which results in approximately two-thirds of the impinging CO_2

laser radiation being absorbed by the outermost 50 u of tissue; heat conduction through the corneal stroma to the aqueous humor of the anterior chamber; cooling of the corneal surface by ambient air; cooling of the aqueous by blood flow from the iris when the aqueous humor is at a higher temperature than that of the blood vessels in the iris; possible ablation of the outermost layers of the cornea on irradiation at higher power densities; increase in the radius of curvature (i.e., flattening) of the cornea at high power densities associated with loss of water and stromalchanges, as discussed in the next chapter, thus narrowing the anterior chamber and bringing the cornea in closer apposition to the lens; and alterations of thermal properties of the cornea including heat capacity and conductivity with injury.

Although all these contribute to the relationship between aqueous and corneal temperature, it is probable that the effect of some of these becomes more significant in certain regions. In the first region of Figure II-4 (low power densities), the temperature of the blood is higher than that of the aqueous humor. In this region, the temperature of the aqueous does not rise greatly, and the predominant effect is probably primarily heat flow from the cornea to the aqueous. There is also possibly little heat lost to the air. In the middle region, the slope is decreased since as the aqueous tmperature elevation rises above that of the temperature of the blood, the vascular supply of the iris tends to dissipate heat from the aqueous, thus tending to minimize the rate of elevation of aqueous temperature. In the third part of the curve, there is not a corresponding increase of the ability of the iris blood supply

to cope with the increased flow of heat into the aqueous. This could be associated in part with an elevation in anterior chamber pressure which could compress the iridial blood vessels. There may be both a decrease in the thermal capacity and an increase in the thermal conductivity of the cornea related to a loss of water from the cornea, and the cornea may be flattened due to loss of water and alteration of the corneal stroma. Consequently, in the third part of the curve, the relative rate of rise of aqueous to corneal temperature will correspondingly increase.

Irradiation of the isolated thermocouple resulted in a lower temperature elevation than corneal surface temperature elevations at the same power density. These data coupled with the results of shielding the thermocouple tip, in which the irradiated corneal temperature fell slowly rather than rapidly, suggests that the surface temperature recordings are not higher than actual. On the contrary, it is entirely possible that the surface recordings are slightly lower than actual because of a temperature gradient between the higher temperature of the cornea and the less absorbant thermocouple. Moreover, because of the small size of the thermocouple, it is possible that the measured temperatures are quite close to actual.

It should be noted that there probably is a temperature gradient across the thermocouple tip in any surface measurement. The effect of this on the temperature recording is difficult to determine. In this case, however, this effect appears to have been minimal.

Increased pressure of the thermocouple on the corneal surface increased the pre-irradiation temperature readings by less than 0.5° C for normal experimental conditions. (Maximal corneal indentation—1.8° C—was not typical of pressures exerted during experimental conditions. This was quickly corrected by a corresponding decrease in the pressure on the corneal surface.) This increased temperature reading may have been partly caused by bringing the thermocouple tip closer to the higher temperature present in the anterior chamber. It may also have been due to decreased exposure of the thermocouple to cooling by the surrounding air and a change in thermal conductivities, since its tip now is somewhat embedded in the cornea. A further factor which must be considered during irradiation temperature recordings is that due to the finite, although large thermal absorption coefficient of the cornea, together with cooling by ambient air, the maximum steady-state temperature elevation of the cornea is not on the surface, but rather deep to the surface. Therefore, a higher temperature would be expected deeper in the cornea, rather than on the corneal surface itself.

Similarly, pressing the thermocouple into the tip of a human finger, which also has a higher internal temperature, resulted in an increased temperature reading of 0.4° C. In contrast, a thermocouple pressed on the cornea of an eye which had been refrigerated for 12 hours and then allowed to approach room temperature for 0.5 hour, showed a 0.5° C temperature decrease with depression of the cornea. In this case, the temperature decrease appears to be caused by bringing the thermocouple tip closer to a lower internal temperature, and/or due to decreased

exposure of the thermocouple to heating by the surrounding air.

Our findings are somewhat different from a previous investigation of infrared (IR) radiation of the eye (Goldmann, 1933b). His radiation source was an arc lamp, the output of which was directed through 2 cm of water and aqueous Lugol's solution before being focused on the corneal surface. With this arrangement, the long wavelength infrared radiation was greatly attenuated. The radiation that reached the eye ranged from a wavelength of 0.9 to 1.4 u. Unlike the output of the CO_2 laser at 10.6 u, these shorter IR wavelengths can penetrate the cornea and aqueous humor (Prince, 1964b). The radiation in Goldmann's studies was absorbed by the pigment of the colored irides. Therefore, in Goldmann's studies, the iris rather than the corneal surface was the primary site of heating.

Our finding that pre-irradiation temperature of the anterior surface of the lens covered by the iris was 0.7° C higher than the exposed lens surface is in agreement with Goldmann (1933a). Goldmann utilized chronically implanted gold, gold-platinum thermocouples for recording internal temperatures, but did not record corneal surface temperatures. He found the temperature under the iris in his irradiated eye was 1 to 3° C higher than the exposed lens surface (Goldmann, 1933b). In contrast, our findings in eyes undergoing CO_2 laser irradiation showed that the temperature under the iris was lower than the temperature of the exposed lens surface (Table II-2). In Goldmann's studies, the higher posterior chamber temperature resulted from the irides being the source of heat (the site of radiation absorption). Our lower posterior chamber temperature resulted from the cooling or "protective" action of the irides.

Hoffmann and Kunz (1934) carried out a series of experiments in which they studied the effects of infrared heating of the eye. They placed a suitably sized cylinder along the optic axis filled with heated water in contact with the cornea of a proptosed eye. The temperature measured was the difference between that of the water and the heated aqueous humor. They heated the proptosed eyes of rabbits with water at temperatures from 47° to 80° C for six to eight minutes and determined the corresponding temperature increases of the aqueous humor in the anterior chamber by means of a thermocouple inserted vertically along the axis of the eye through the cornea. They reported that the epithelial cells became temporarily hazy when heated to 48° C and remained cloudy after being heated to 50° C. The cornea became permanently cloudy when heated to 65° C. The iridial vasculature became increasingly narrowed as the temperature of the aqueous humor increased; the iridial arteries were generally empty whereas the veins were enlarged. The capillaries in the deeper iridial layers were alternately enlarged and constricted so the appearance was that of small sausages. There were granular regions of the subepithelial lens fibers when the aqueous humor temperature was increased to 46°C; however, the lens epithelium appeared intact. When the temperature of the aqueous humor was increased above 46° C, a thick, white subcapsular layer was produced in the lens. They noted the comparatively increased damage upon heating an enucleated eye, and commented upon the cooling abilities of the ocular circulation.

Some of Hoffmann and Kunz's temperature data are in general agreement with that in this investigation (Table II-5). Comparisons of all

their data with the results in this investigation are difficult, how-ever, since they heated the corneas directly with a water-filled chamber and did not necessarily maintain the temperatures within this chamber constant during the six to eight minutes of exposure.

CHAPTER III

PRESSURE AND TEMPERATURE DETERMINATIONS IN THE ANTERIOR CHAMBER OF THE RABBIT EYE DURING CO_2 IRRADIATION OF THE CORNEA

Fine, Zimmerman and Fine (1966) and Fine, Fine, Peacock, Geeraets and Klein (1967) reported the presence of concavities of the anterior surfaces of lenses of eyes irradiated at 15-50 W/cm^2 for one second, then excised and fixed. The unwhitened indentations were often present following high power, non-perforating exposure of the corneas. Indentations were always present, but were whitened, following exposures which perforated the cornea, in these rabbit eyes.

Geeraets, Fine and Fine (1969) reported additional investigations on the lens indentation following non-perforating CO_2 laser irradiations of the cornea. The power densities and exposure times ranged from 9 to 20 W/cm^2 and 1 to 4 seconds respectively. The initial corneal scarring and opacities resulting from these irradiations made inspection of the lens in vivo nearly impossible. They reported the appearance of anterior lens indentation , however, in one living rabbit eye nine months after irradiation by slit lamp examination through the clear peripheral portion of the cornea central to the limbus.

The absorption of CO_2 laser radiation by the cornea has been discussed in Chapter II. Only a negligible percentage of the original energy impinging on the cornea could have been transmitted through a 500 u thick cornea. Even with ablation and penetration of the cornea, a thickness of aqueous humor, 3 millimeter maximum thickness, may separate the posterior surface of the cornea and the anterior surface of the lens.

The absorption coefficient, α , of the aqueous humor at 10.6 u is proably more similar to that of sea water (α approx. 750 cm^{-1}) than to that of tissue and would result in an even greater attenuation of the radiation. Therefore, during non-perforating CO_2 laser corneal irradiation, essentially no 10.6 u energy would impinge on the lens surface.

During carbon dioxide laser corneal irradiation, the temperature and possibly the pressure of the anterior chamber may be increased. Data from Chapter II indicated that the aqueous humor temperature is increased. The eye is essentially a closed, filled cavity; the volume of the anterior chamber is small (0.25 cc in the adult rabbit eye). Elevated aqueous humor temperatures together with an alteration in corneal curvature may increase the intraocular pressure. This pressure elevation might not be maintained since an increased flow rate through the trabecular region may occur in response to increased intraocular pressure, and the alteration in the corneal curvature might not be permanent. It would therefore be of interest to determine the pressures associated with steady-state threshold and suprathreshold CO_2 laser irradiations of the cornea. Furthermore, since lens indentation has been produced without corneal perforation on pulsed CO_2 corneal irradiation, it would be of interest to measure the intraocular temperature and pressure elevations associated with pulsed relatively high power density irradiation of the cornea, and to determine the relationship between these parameters and the lens indentation.

The avascular cornea and lens are dependent for normal metabolism in part on the aqueous humor produced by the ciliary processes and irides;

an alteration in metabolism could result in lessened optical clarity. Increased temperature and pressure caused by irradiation of the cornea could injure the ciliary processes and irides and thereby permanently alter aqueous humor production and composition. An altered aqueous humor composition might impair lenticular and corneal metabolism and decrease the optical clarity of these tissues.

Determination of the alteration in all aqueous humor components would be difficult since a large number of substances are normally present in relatively small quantities. According to Davson (1969), however, irritation of the iridial vasculature can result in a breakdown of the blood-aqueous humor barrier, thus producing an increase of protein in the aqueous humor. Evans blue is an innocuous dye with a great affinity for serum proteins. Injected intravenously, it will indicate the presence of any extravasated plasma proteins. After an intravenous injection of Evans blue, any increased flow of plasma protein into the aqueous humor following CO_2 laser irradiation of the cornea should tint the anterior chamber a characteristic blue. Also, soluble protein can be measured by UV spectroscopy; this technique requires the removal of aqueous humor but is not destructive to the sample.

Therefore, the purposes of this chapter are: 1) to determine the temperature and pressure in the anterior chamber during and immediately following both continuous suprathreshold unfocused and pulsed focused CO_2 laser irradiation of the cornea at the power levels we had available, and to determine the conditions of temperature and pressure under which lens indentation occurs; and 2) to investigate the effect of non-perforating

laser irradiation of the cornea on the increase of aqueous humor protein as a possible reflection of its effect on the blood aqueous barrier.

Methods and Materials

Laser

The laser used in these experiments is described in Appendix 1.

Low power density irradiation

Low power density (100 to 350 mW/cm^2) irradiations of the cornea were carried out as described in Chapter II.

High power density irradiation

Since the power output of the laser was about 10 watts, higher power density outputs were obtained by focusing the beam through a specially constructed focusing cone, designed for use with the CO_2 laser beam. Diagrammatic views of this device are shown in Figure III-1. It had been turned out on a lathe from a 25 x 60 millimeter outside diameter aluminum rod. The center was a truncated cone whose axis coincided with that of the aluminum rod. The conical inner walls had been coated with gold in a vacuum diffusion chamber to increase the reflectivity of the unit.

In use, the cone was aligned so that its axis coincided with that of the laser beam directed at the entrance port (base of the cone). Radiation entering the cone was internally reflected forward through the exit aperture (Mendenhall wedge effect). The greatest portion of the

entering energy was collected in a 5 millimeter diameter area approximately 5 millimeters distal to the exit port of the cone (the truncated portion of the apex of the cone). It was calculated that the energy density in this focal region would be greatest at the center (for a gross approximation of the energy density distribution of the beam in this region see Figure III-2). In order to align the cone a section of Thermofax paper was held approximately 20 millimeters distal to the exit port—when the axis (laser beam and cone) were aligned, a bulls'-eye pattern was produced, the outer ring of which was considerably less intense than the central spot. If improperly aligned, the inner spot produced was eccentrically located.

The power output at the exit of the cone was maintained at 6 to 7 watts during irradiation. Pre-irradiation measurements were made with a Coherent Radiation Model 201 detector.

Pressure Measurement Unit

Mercury Manometer Used for Observing Pressure Elevation

Initial anterior chamber pressure measurements were made by means of a mercury manometer. This device is shown schematically in Figure III-3. Intraocular pressure changes were measured by connecting the anterior chamber via a hypodermic needle to a closed system of normal saline abutting a mercury reservoir and a one millimeter inside diameter capillary tube. For added sensitivity the manometer capillary tube axis was at an angle of 45 degrees to the vertical.

For calibration of the manometer a strip graduated in millimeters was attached parallel to the capillary tube. The manometer system

needle was inserted into the lumen of a section of Tygon tubing filled with normal saline. The mercury column was calibrated at 0 mm of mercury (ambient atmospheric pressure), 20, 50, 75, and 100 mm of mercury based on heights of 0, 27.2, 68,102,and 136 centimeters of normal saline respectively (the specific gravity of normal saline is approximately unity).

The Modified Mercury Manometer System Used for Measuring Intraocular Pressure

In order to record the transient pressure pulse this manometer was modified as follows (Figure III-4): a tungsten wire (5.51 ohms/cm at 20 C) was threaded into the mercury column in the capillary tube; a stainless steel wire (0.007 inches diameter) was inserted through the Tygon tubing into the manometer saline reservoir. A potential difference of 22 volts was applied between the tungsten and stainless steel wire from a power supply (Sorenson Q Nobitron QR 40). Variations in the height of the mercury column altered the resistance in the circuit by changing the exposed length of the tungsten wire. The electrical output from this pressure measuring device was recorded using a Massa Cohu amplifier recorder (PR 201) or an oscilloscope fitted with a Polaroid camera.

Statham Transducer System Used for Recording Intraocular Pressure

In the major phase of this investigation, the mercury manometer was replaced with a Statham P32 AA pressure transducer connected by means of a short section of Tygon tubing to the glass tubing. The system was filled with normal saline, containing 100 units of heparin/100 ml. to prevent blockage of the hypodermic needle sealed into the anterior

chamber. Outputs from the transducer as well as the amplified output from the thermocouple were recorded on separate channels of a Gilson Polygraph (Model M 8 PM). A diagrammatic representation of the set up is shown in Figure III-5. Although the transducer was equipped with a self-contained pressure calibration, the device was also calibrated with a column of saline as previously described.

Temperature Measurements

A thermocouple (chromel-constantan) (TC-RC-IT-200 BLH) was used to measure the temperature of the aqueous humor. The associated equipment and circuitry required to produce recordings of temperatures from the thermocouple have been described in Chapter II. The calibration procedure was described in the same section.

Simultaneous Pressure and Temperature Measurements in the Anterior Chamber During CO_2 Laser Irradiation of the Cornea

In early experiments attempts were made using the modified mercury manometer (Figure III-4) and the thermocouple to make simultaneous pressure and temperature recordings during a CO_2 laser corneal irradiation. The output of the manometer circuit was connected to a dual trace oscilloscope or to separate channels of a Massa Cohu PR 201 recorder. When pressure changes were measured with the Statham transducer, the output of this device and that of the thermocouple were recorded on separate channels of a multichannel Gilson Polygraph.

Animals

Over 200 male and female albino rabbits (5 to 10 pounds) were used in this study. Each animal was systemically anesthetized with an intramuscular injection of Innovar Vet (fentanyl 0.4 mg/ml, droperidol

20 mg/ml,McNeil). The volume of Innovar Vet required for adequate anesthesia varied from 0.1 to 0.2 ml per pound of body weight. Atropine sulfate (0.02 to 0.06 milligrams per pound) was administered intravenously to control respiratory distress. The eyes were anesthetized by the instillation of Ophthetic drops (proparacaine HCl-Allergan) approximately five minutes prior to exposure. The pupils were dilated when necessary prior to irradiation. In earlier trials a solution of 2% atropine sulfate was used. In later cases, however, a 1% solution of Mydriacil (bis-tropicamide, Alcon) was employed.

Prior to irradiation each animal was placed in a rabbit box fitted with a chin rest. The head was immobilized in position with tape and the eyelids held apart with a metal speculum.

Technique for In Vivo Pressure Measurements in the Anterior Chamber During CO_2 Laser Irradiation of the Cornea

For pressure measurements in the living eye (with either the mercury manometer or the Stathan transducer) the anesthetized, immobilized rabbit was positioned so the axis of the irradiating beam and the axis of the eye approximately coincided (Figure III-6). The animal was positioned 5 mm distal to the exit port of the focusing cone. Proper alignment of the position of the eye could be made prior to irradiation by sighting down the axis of the cone. The eye was gently proptosed, stabilizing with the aid of pressure from a cotton-tipped applicator and the horizontally-held manometer needle rapidly inserted through the cornea slightly anterior to the limbus. During the insertion, the iris, lens and posterior surface of the cornea were avoided. Once in place, the needle was sealed to the corneal surface with Eastman 910 adhesive

(methyl cyanoacrylate). The eye was released from proptosis and the lids held open with a metal speculum. The aqueous humor pressure was adjusted to 20 mm of Hg (above atmospheric pressure) and sealed by means of a clamp on the manometer Tygon tubing. The cornea was then irradiated and the maximum excursion of the mercury column observed and recorded.

Simultaneous Recordings of Pressure and Temperature in the Anterior Chamber During CO_2 Laser Irradiation of the Cornea

Attempts were then made to record concomitant pressure and temperature variations in the anterior chamber during corneal irradiation. To accomplish this, the manometer needle was inserted and sealed into the proptosed eye as described above. The aqueous humor pressure was adjusted to 20 mm Hg, and the thermocouple inserted through a second corneal puncture made by a 24 g hypodermic needle. This insertion site was anterior to the superior portion of the limbus. The thermocouple was positioned so the heat sensing tip was about 1 mm anterior to the center of the anterior surface of the lens. It was immobilized and then sealed to the cornea. Following this procedure the intraocular pressure was readjusted to 20 mm Hg

The eye was positioned so the axis of the beam was coaxial with the underlying thermocouple tip. In certain instances, the irradiating beam was shifted so the axis was displaced laterally by approximately 3 mm.

Pressure and temperature were measured simultaneously in eyes during either continuous low power density irradiations of the entire corneal surface or pulsed high power irradiation of a 5 mm diameter area of the center of the cornea. The lower power density irradiations ranged from 100 to 350 mW/cm^2 for 10 to 20 minutes. The high power density

irradiations were all carried out with an average power density of about 30 to 35 W/cm^2, i.e., 6 to 7 watts focused through the cone on a 5 mm diameter area. The pulse length of the majority of these irradiations ranged from 0.5 to 1.75 seconds. Some eyes were irradiated continuously until the corneas perforated.

Observation of the Alteration of Corneal Curvature During Laser Irradiation

The corneal surface was observed in several eyes during irradiation of the center of the corneal surface with the focused CO_2 laser beam (7 watts for one second). The corneal surface was viewed at right angles to the optic axis of the eye against a section of millimeter-ruled graph paper in order to estimate the distance the cornea moved during irradiation. Also, in order to record any corneal surface displacement, photographs were taken prior to and immediately post irradiation.

The irradiated eyes were enucleated and dissected immediately to determine the distance between the posterior surface of the cornea and the anterior surface of the lens.

Investigation of the Effect of Heat on the Lens Cortex

In order to investigate the effect of heat upon lenses the following experiment was carried out: The lens was removed from enucleated unirradiated eyes by cutting the zonules. It was decapsulated by pulling the corner of the centrally-incised posterior capsule laterally and anteriorly. The decapsulated lens was then placed on its posterior surface, a stream of heated water (70° C, 10 ml in a glass pipette) was directed to the mid-anterior surface. Similar treatment was carried out on different control lenses with water heated to 40° C. The appearance of the lens was observed and recorded, then placed in 3% glutaraldehyde.

Certain eyes were irradiated over the mid-cornea at 7 watts focused for 1-1/2 seconds and then enucleated. The lenses were dissected and decapsulated as described above, then examined.

Increased Protein in the Aqueous Humor Following Non-Perforating Irradiation of the Cornea with a CO_2 Laser Beam

Qualitative Determinations with Evans Blue

Prior to irradiation, 1.5 to 2.0 ml of 10 percent solution of Evans blue was injected intravenously into six animals via the marginal ear vein. This was done prior to irradiation in all animals in this group and also at intervals post irradiation in a few of these animals. Observation of the dye in the irradiated and the contralateral control eye was noted visually. Spectrophotometric determinations of Evans blue concentration were done on dilutions of aqueous aliquots sampled with a 50 microliter Hamilton syringe.

Quantitative Determination—UV Spectroscopy

Protein concentration determinations were made of paired control, and irradiated and contralateral control aqueous humor. Determinations were made by one or more of the following spectrophotometric methods: 1) the Kalckar modification (1947) of the method of Warburg and Christian. In this method (1.45 times the optical density at 280 nm) minus (0.85 times the optical density at 260 nm) is equal to the grams percent protein. 2) The method of Waddell (1956). In this method the difference between the O.D. at 215 nm and that at 225 nm is multiplied by a factor (144 for the Beckman DU Spectrophotometer); the product is micrograms of protein per milliliter of solution (ug/ml). Bovine serum albumin standards as reference were concomitantly assayed.

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The left eyes of a group of two sets (13 albino rabbits) of apparently identical litter mates were irradiated at 6 watts for 1.5 seconds; the animals were routinely anesthetized and their exposed eyes irradiated with the cone on one of the following sites: (A) the corneal surface over the center of the well dilated pupil; (B) the corneal surface over the iris adjacent to the pupil; (C) the corneal surface over the mid-radius of the iris; (D) the scleral surface over the ciliary muscle region (approximately 2 millimeters posterior to the limbus). Only the eyes irradiated over Site A were dilated with Mydriacyl prior to irradiation. One pair of eyes were not irradiated and served as controls. Eyes were sampled immediately post irradiation or at one or two days after exposure. Each eye was proptosed and sampled once with a 50 uL Hamilton microliter syringe inserted into the anterior chamber. The aliquot of aqueous humor was diluted with 30 ml of normal saline and the optical densities determined at 215 and 225 nm, and at 260 and 280 nm on the Beckman DU spectrophotometer. Bovine serum albumin standards were determined concomitantly. The animals were killed (after sampling); the eyes were enucleated and fixed in 3% glutaraldehyde. Each fixed irradiated eye was dissected and examined under a dissecting microscope. In this manner the location of the irradiation could be verified by changes in the cornea and underlying iris.

Results

Initially, maximum pressure elevations were gauged visually using the mercury manometer on irradiation. The range of observed intraocular pressure peaks varied considerably; during 1/2 to 1 second irradiations the maximum pressure elevations observed ranged from 14 to 30 mm Hg

and from 20 to 60 mm Hg, respectively. Numerous permanent recordings of the pressure pulse were obtained with the amplifier-recorder and with the oscilloscope-camera arrangement using the tungsten-wire technique, but the results were disappointing. The recordings indicated a period of pressure increase which approximately coincided with the duration of irradiation pulse; however, the peak values recorded were approximately one-half the pressure rise observed visually.

Simultaneous temperature and pressure recordings could not be obtained with the tungsten-wire system. There appeared to be an interaction between the electric systems. Because of the inaccuracies and problems, pressure measurements as discussed belowwere therefore carried out with the Statham transducer.

Low Power Density Irradiations of the Entire Corneal Surface

No pressure rise was detected in the anterior chamber on 10-minute irradiation of the entire cornea at 0.1 w/cm^2. The temperature in the anterior chamber rose 4° C after three minutes, plateauing at this level on irradiation at this power density. As the power density was increased, the aqueous humor temperature increased. At 0.15 w/cm^2 the temperature increased, plateauing at approximately 5° C and increased no more after three minutes of irradiation. At 0.35 w/cm^2 there was a gradual increase of pressure, plateauing at 40 mm Hg in 18 minutes with a temperature rise of about 12° C, which remained constant after six minutes. At higher power levels of irradiation, the maximum pressure elevation reached in the anterior chamber with unperforated corneas depended, within limits, upon the duration of the pulse.

High Power Density Irradiation of the Mid-Corneal Surface

On short pulse duration, high power density irradiation, the pressure and temperature in the anterior chamber increased concurrently; the following are representative of the data obtained.

In Figure III-7, A; the eye had been irradiated at 6 watts for 1.5 seconds with a beam focused through the cone at the center of the anterior corneal surface. The pressure in the anterior chamber rose steadily from 20 to 79 mm Hg at the end of the pulse. Following cessation of the pulse, the pressure fell to 40 mm Hg in 10 seconds, then fell gradually to 30 mm Hg during the next 30 minutes. In the living eye, respiratory and arterial pulses were always readily observed. For temperature measurements, the thermocouple was placed in close approximation to the center of the anterior surface of the lens (i.e., in front of the pupil) along the axis of corneal irradiation. The temperature rose to 73° C at the end of the pulse and returned to base-line levels (∿ 35° C) in several minutes. Peripheral displacement of the thermocouple resulted in a lower, delayed temperature rise. For example, when the thermocouple tip was located 3 mm from the axis of the irradiating beam, the maximum temperature elevation was approximately 10° C.

When irradiation was carried out at approximately the same power levels for 0.75 seconds (Figure III-7, B), the pressure and temperature elevations were correspondingly less—to 66 mm Hg and 49° C.

Changes in pressure and temperature were similar on irradiation of eyes in dead animals (Figure III-7, C), except for loss of the respiratory and vascular pulses. One minute following the peak pressure, the percent increase of peak pressure in live eyes was significantly higher

than in eyes of dead animals (29.6 percent $\pm$ 2.2 S.E.M., compared with 18.9 percent $\pm$ 2.1 S.E.M.).

In all eyes in which the corneas were perforated, the pressure dropped rapidly to zero.

Gross observations and dissections were carried out on both eyes from animals irradiated in this portion of the study. This was done not only on animals whose anterior chamber pressure and temperatures had been monitored but also on similarly irradiated eyes in which these parameters had not been measured. Both irradiated and control eyes were enucleated immediately post irradiation; certain eyes were examined without fixation, others were dissected following fixation in glutaraldehyde.

Appearance of Eyes

The appearance of the corneas after low power (100 to 350mW/cm^2) continuous irradiation has been described in Chapter II. No gross internal alterations were observed in these eyes.

The corneal site of cone-focused (6-7 W) irradiations was immediately whitened by exposures lasting more than 0.5 seconds. The diameter of the corneal lesion increased as a function of exposure time. For example, the diameter was 1 millimeter after a 0.5 second exposure and about 2 millimeters on a 1.75 second exposure. Furthermore, irradiation pulses of 1 second and longer ablated the center of the irradiated area. (See Figure III-8).(Note that this eye had been monitored for pressure and temperature.) A 1.5 second irradiation produced a 1 mm diameter crater whose depth was approximately one-half the thickness of the cornea; blackened material was observed on the walls of the crater.

After 3 seconds of focused irradiation (6-7 watts) the corneas perforated and heated aqueous humor was expelled.

Dissection of corneas showed that the irradiated site was thickened—thickening of the tissue immediately adjacent to the axis of the irradiating beam increased as the pulse duration increased.

Dissection also revealed that the anterior chamber depth had decreased markedly following 1.5 to 1.75 second focused irradiations of the cornea. The posterior surface of the corneas appeared to be in contact with the anterior iridial surfaces and was close to the anterior lenticular surface. This was observed in both unfixed and fixed eyes.

The relative distance between the cornea and the anterior lens surface was monitored during irradiations of several eyes. By the termination of a focused 7-watt irradiation the radius of curvature of the cornea appeared to have increased; the anterior surface was displaced approximately two millimeters posteriorly. As a result the anterior chamber depth appeared to be decreased temporarily (Figure III-9).

Lenses from the unfixed excised (irradiated and control) eyes were examined following reflection of the corneas or dissection from the eyes. Examinations were made by oblique illumination under a dissecting microscope. The anterior surfaces showed a normal shagreen and were smoothly curved. In eyes in which the corneas had perforated during irradiation there was a small (approximately 1 millimeter in diameter) whitened area coaxial with the site of perforation on the anterior surface of the lens but there was no sign of an indentation.

Certain of these unfixed lenses were decapsulated as described. The capsules were removed readily from all control lenses without the

obvious adherence of any cortical material and the contour of the exposed anterior surface of the lens remained smoothly convex. In contrast, decapsulation of unfixed lenses from eyes following focused irradiations with 6-7 watts for 1.5 seconds or longer did not result in such complete separation of capsule and underlying lens material on strippage. A region of subcapsular material coaxial with the site of corneal irradiation remained attached to the posterior of the capsule. Apparently it had pulled away from the remainder of the lens cortex. The surface of the resultant crater was irregular; the diameter was approximately 2 mm.

Eyes fixed in glutaraldehyde or formalin following irradiation and enucleation were also examined. The appearance of the site of irradiation of the cornea was not remarkably different from that observed in unfixed eyes. The anterior lens surface of all eyes irradiated for 1.5 seconds or longer through the cone, then excised and fixed, however, showed indentations (Figure III-10) which appeared to be coaxial with the corneal lesions. These pulses had raised the temperature near the lens surface to 60 or 70° C. The diameter of these indentations, 2-3 millimeters appeared to have increased with increasing pulse length. The indentations from perforated eyes were whitened. Indentations were still demonstrable in lenses from eyes excised and fixed in glutaraldehyde or formalin nearly one year following focused irradiation (6-7 watts, 1.5 seconds or longer). Indentations were never found in lenses from eyes that had been similarly fixed but not irradiated, or from eyes irradiated for shorter periods of time (i.e., with pulses of power and time insufficient to raise the underlying aqueous humor to a temperature greater than 60° C).

Indented and contralateral control lenses from eyes excised immediately post irradiation were stained by immersion in 1% acid orcein, after fixation. The indented areas stained a light yellow, the remainder of the lens as well as the entire control lenses were stained an even maroon color.

Lenses were dissected from unirradiated eyes and the center of the anterior surfaces were heated with water (approximately 70° C) as described. When these lenses were decapsulated, the cortical material underlying the previously heated area adhered to the capsule. The exposed lens cortex had a ragged crater which appeared similar to those observed when lenses from irradiated eyes were decapsulated. When lenses were heated in this manner following decapsulation, a smooth indentation appeared immediately. (This did not occur when decapsulated lenses were treated with water at 40° C.) These indentations in heated decapsulated lenses were present without fixation; when these lenses were fixed in glutaraldehyde or formalin the indented region appeared slightly larger.

Lens indentations were never observed in material fixed in 100% ethanol following irradiation—regardless of the pulse length. Examination revealed that the lens capsule was slightly irregular in a circular region (approximately 2 mm diameter), however, which appeared to be coaxial with the corneal irradiation site.

Investigation of the Protein Concentration of the Aqueous Humor Following Irradiation of the Cornea with the CO_2 Laser Beam

Qualitative—Evans Blue

When a non-perforating CO_2 laser suprathreshold irradiation was directed at a region of the cornea anterior to the iris, the following

events were noted in animals previously injected (I.V.) with Evans Blue. In the irradiated eye, the anterior chamber immediately became a deep blue. Also, the tears in these eyes were blue, presumably from traumatization of the conjunctival vasculature by the speculum. Injections of Evans Blue two days following the irradiation still resulted in coloration of the aqueous humor. Only slight coloration of the aqueous humor was observed following focused irradiation of the cornea over the center of the well-dilated pupil. In the contralateral control eyes neither the anterior chamber humor nor tears were colored.

This technique was not utilized to quantitate the increased aqueous humor protein study because of the lack of sensitivity. Following necessary dilution of the aliquot (for the darkest of aqueous humor samples) with normal saline, the resultant optical density value at 750 nm (where there appeared to be an absorption peak for Evans blue) was very small.

<u>Quantitative Determination—UV Spectroscopy</u>

The modified method of Warburg and Christian (Kalckar, 1947) was not suitable for the determination of aqueous humor protein levels in diluted samples from individual control eyes in this study. The method could be utilized only for determination of protein in aliquots from eyes which had suffered the most damage (e.g., irradiation of the cornea over the mid-radius of the iris). Furthermore, some of the samples from irradiated eyes had optical densities at 260 nm which were greater than at 280 nm. (Use of such data with the formula of Kalckar would have given negative values.)

Using the method of Waddell (1956), protein was determined in the aqueous humor of control eyes and those irradiated at various sites

(Figure III-11). The protein levels of control eyes, 60 mg%, were similar to those reported by others (Davson, 1969) of 50 mg%. The height and duration of the protein elevations in irradiated eyes depended on the location of the irradiation site and the interval between irradiation and sampling. In these experiments, each eye was sampled only once. The maximal aqueous humor protein elevation occurred following irradiation of the cornea over the iris at Site C (Figure III-11). Initially, the protein concentration increased to about ten times normal one day post irradiation (636 vs 60 mg%). The concentration decreased slightly on the ensuing day. The elevation following irradiation of the cornea at Site B (i.e., the corneal surface directly over the edge of the pupil) rose gradually to a maximum (approximately 350 mg%) two days post irradiation. Irradiation over Site D (sclera over the ciliary body) resulted in a slight increase of aqueous humor protein which continued to rise in the day following irradiation. The two-day sample of this group was lost. Irradiation of the cornea at Site A (the corneal surface directly over the center of the well-dilated pupil) did not increase the aqueous humor protein concentration during the two days following irradiation. These results suggest that the increase of intraocular pressure per se did not result in damage to the blood aqueous barrier. Temperature as well as pressure elevations appeared to be involved in this injury.

Following sampling, anesthetized animals were killed, the eyes enucleated and fixed in glutaraldehyde. Gross dissection of the fixed eyes were carried out. Hemorrhagic regions of the iris or ciliary body vasculature verified that the expected areas were actually injured on

irradiation. The eyes irradiated at Site A contained lenses with indentations of the anterior surfaces. Lenses from eyes irradiated at Site B and C showed some irregularities of the anterior lens surfaces in the respective regions.

Discussion

The absorption coefficient for tissue at 10.6 u has been determined as 150 to 270 cm^{-1}. The approximate values for the adult rabbit eye cornea and anterior chamber depths (along the visual axis) are 0.05 and 0.3 cm, respectively (Prince, 1964a). The radiation transmitted to the lens surface can be estimated by the formula $I/I_0 = e^{-\alpha x}$, where α = the absorption coefficient for tissue or water (aqueous humor) and x = the distance in centimeters. For example, it was estimated that less than 1% of the radiation incident on the corneal surface would be transmitted to the posterior surface of the cornea, utilizing the lower value of α for tissue. Therefore, even if only a part of the cornea were intact, it is apparent that the percentage transmitted through the 0.3 cm of aqueous humor to the lens surface would be extremely small and thus non-perforating CO_2 laser corneal irradiations should not be hazardous to the lens. Experimental data, however, indicated that this assumption was incorrect. Anterior lens indentations can be produced by non-perforating CO_2 laser irradiations of the cornea. On lateral observation of the anterior portion of the living eye and on dissection of enucleated eyes, it appeared that the depth of the anterior chamber had decreased markedly post irradiation. At this time the posterior corneal surfaces were in close approximation to the anterior iridial and exposed pupillary lenticular surfaces.

The interior of the eye may be regarded as a closed, filled cavity. During irradiation the temperature of the cornea and of the aqueous humor increased, resulting in some intraocular pressure elevation.

When the collagen fibers in the stroma reached their critical shrinkage temperature there was increased tension within the corneal stroma and the cornea attempted to flatten. This caused a further pressure increase. When it became greater than the blood pressure, it prevented blood flow into the iridial arteries and expressed blood from the iridial veins. The formation of aqueous humor probably was decreased. Similarly, the increase in pressure in the aqueous humor should have increased the outflow. Consequently, there probably was a decrease in aqueous humor volume coincident with flattening of the cornea. Also, the viscosity of water decreases as the temperature is increased (Handbook of Chemistry and Physics, 1963). The combination of increased pressure and a lessened viscosity (following heating during corneal irradiation) may have permitted an extraordinarily rapid percolation of aqueous humor through the trabecular region. This rapid efflux may have occurred with little or no tearing. It is also possible that in addition to the increased pressure and decreased viscosity, there may have been an alteration in the trabecular meshwork region. Indeed, it may have torn. If it were torn, however, the pressure in the eye may not have returned to normal following irradiation. That is, the cornea would not have returned to its normal curvature, as was observed.

There was a protracted elevation of the intraocular pressure observed in eyes of live versus dead animals following irradiation. This probably resulted from the difference in intraocular blood flow. A greater

portion of blood was forced from either eye during the time when the intraocular pressure exceeded the intraocular blood pressure. Obviously following irradiation the blood flow was never re-established in the eyes of dead animals; hence there was less force maintaining the intraocular pressure elevation.

On CO_2 laser irradiation, the outermost layers absorbed the greatest percentage of the impinging radiation. During irradiation of the cornea, convection and conduction by air and conduction to adjacent tissue will remove a portion of the heat from the irradiated regions. In the present investigations, it is possible that the site of maximum temperature was not at the corneal surface, but deep to it, perhaps in the stromal layers. The corneal stroma, which comprises 90% of the thickness of the cornea, has a high collagen content. The collagen is in the form of bundles of fibers which are somewhat parallel to the corneal surface (Davson, 1969). These fibers are composed of collagen fibrils which consist of collagen molecules (Verzar, 1963). The collagen molecule is a triple helix with stabilizing cross-links. During heating to a specific temperature, areas of these cross-links are broken. The fibrils are no longer maintained in linear array. The resultant random pattern results in a local shortening. Consequently, when the temperature of the stroma was increased to the critical shrinkage range (65-70° C), the length of the collagen fibers suddenly may have decreased to as much as one-third of the original length.

This sudden shortening of the stromal fibers during irradiation apparently acted as a drawn "purse-string," causing a temporary shortening of the corneal arc-length. The resultant increase in the corneal

radius shortened the depth of the anterior chamber and brought the posterior surface of the cornea in close apposition to the anterior lens surface and iris. Consequently, the temperature of the aqueous humor was elevated by heat flow from the cornea and perhaps to an extent, by the laser radiation as the cornea was ablated.

The heated posterior corneal surface under the irradiated area and its adjacent heated aqueous humor was temporarily pressed against the exposed lens surface. Consequently, the temperature at the lens surface would have been increased further. There was a concomitant pressure increase in the anterior chamber.

According to Dische (1970), the collagenous lens capsule also exhibits heat shrinkage. Histologically, the capsule, epithelial layer, and adjacent fibers appear to be attached at each interface. Judging from the intercalcated movement exhibited during accommodative processes, they may all move as a unit under pressure. The contents of the lens fibers appear to be somewhat fluid; during the transient shrinkage of the lens capsule during heating, the ends of the subjacent attached lens fibers would also be subjected to compression and temperature increases.

On irradiation, pressure on the fibers in the region of the indentation (as observed on fixation), together with heating of these fibers, may have permanently altered them. This alteration was not evident in the unfixed encapsulated lens. This may, in part, have been due to the fact that the fibers still remained attached to the epithelial cells and capsule in this region, and the capsule appeared to resume its normal curvature. The observed indentation may have been due to fixation of these attached fibers underlying this region, previous to fixation-hardening of the overlying capsule. The fibers may have been effectively

shortened in this region on fixation, in contrast to the fibers in the remainder of the lens. The apparent protein denaturation of these fibers could be exhibited on fixation immediately following irradiation and was still demonstrable in lenses from eyes sampled months after irradiation. The altered staining characteristics of the indented area of fixed lenses suggest some tissue alteration. Visualization of the (*in vivo*) lens following non-perforating CO_2 laser corneal irradiation is extremely difficult because of the resulting opacities in the cornea. Geeraets, Fine and Fine (1969) reported, however, that they could see on slit lamp examination indentated areas of the anterior lens surface in an intact living eyes following non-perforating CO_2 laser irradiation of the cornea. This indentation was not observed in our unfixed excised lens; apparently the lens capsule exhibited sufficient tension to prevent local cortical indentation. This tension apparently was overcome in the intact eye by the intraocular pressure, in the *in vivo* observation.

In the normal unfixed lens, the capsule and epithelial layer can be readily separated from the underlying cortex. In contrast, in these investigations, it was difficult to separate the capsule clearly from the underlying cortex in the region where the indentation would occur. The fibers in this region tore and remained attached to the capsule. Either the fibers themselves were more brittle or more strongly attached to the capsule in this region. Consequently, the fibers in this region were altered, although the alterations were not evident on gross examination of the intact lens.

The application of 70° C water with pressure to a small region of the decapsulated normal lens resulted in shrinkage of fibers in this area, without whitening. This concavity was probably visible without

fixation because of the lack of outward pull by the capsule on the affected cortical area. Furthermore, it indicated that the fibers themselves are altered by the combination of temperature and pressure.

The mercury manometer measurements of the transient intraocular pressure changes during irradiation were similar to but less than those later determined by the use of the Statham P32 AA transducer. The lower values determined from the excursion of the mercury manometer-height resulted presumably from inertia in the system because of the high specific gravity of the mercury (13.6). In addition, recordings attempted by electrically recording variations in the height of the mercury column never reflected the maxium Hg column excursion observed during irradiation. This lack of recording sensitivity may have resulted in part from the poor electrical contact between the mercury column and the immersed tungsten wire. Tungsten and mercury can form an amalgam. This amalgam might have caused a local increased resistance at the interface of the two elements in this system.

Irradiation of the cornea directly over the mid-pupillary region did not increase the aqueous humor protein concentration, whereas irradiations of the cornea directly over the middle section of the iris resulted in a maximal aqueous humor protein increase. Assumedly the transient pressure increase in each of these irradiations, however, were similar. The temperature-elevation may have been the critical factor; the blood-aqueous humor barrier was damaged only when the iridial vasculature was transiently heated to 60 up to 70° C.

Aqueous humor protein determinations suggested that transient temperature increases of the aqueous humor resulted in a marked increase of

aqueous humor protein concentration. Presumably, the blood was the source of the protein, as irradiation over highly vascular areas resulted in massive increases of aqueous humor protein, whereas irradiation over the widely dilated pupillary area did not alter the aqueous humor protein concentration. This is in agreement with the finding of the Evans blue investigations; no color was observed in the aqueous humor following focused irradiation over the pupillary region. Furthermore, aqueous humor protein increases were accompanied by a hemorrhagic area in the vasculature underlying the irradiated area. Following injury midway between the pupillary margin and the root of the iris, the protein concentration continued to increase during the day following the irradiation. Irradiations of this region of the cornea over the iris probably thermally injured the vascular ciliary processes on the posterior surface.

CHAPTER IV

ALTERATIONS IN THE MORPHOLOGY, IN THE ELECTROPHORETIC MOBILITY OF CRYSTALLINS AND IN LENTICULAR ASCORBIC ACID AND REDUCED GLUTATHIONE FOLLOWING IRRADIATION OF THE CORNEA OF THE WEANLING ALBINO RABBIT WITH THE CARBON DIOXIDE LASER BEAM

Data from Chapter III indicated that lens indentations occurred in the course of transient corneal flattening during the irradiation; during this period the depth of the anterior chamber of these adult rabbit eyes was temporarily decreased and the underlying heated aqueous humor was brought in close apposition to the lens surface. These unwhitened lens indentations were permanent, that is, they were still demonstrable in eyes which were excised and fixed in glutaraldehyde one year post irradiation. In general, these indented areas were not whitened and in no instance were cataracts, either partial or complete, observed.

The material in the unwhitened indented regions of fixed lenses from irradiated eyes appears to have been immediately and permanently altered. That is, the indentations were demonstrable immediately post irradiation and at periods up to one year following the exposure. The presence of the indentations implies that lens proteins in the region beneath the irradiated cornea may have been denatured without visible coagulation. If the proteins in the indented region had been denatured without coagulation, their electrophoretic pattern might have been altered (Joly, 1965). Therefore, it would be of interest to determine whether an alteration would be produced in the electrophoretic pattern of the soluble proteins of lenses irradiated with sufficient intensity to produce lenticular indentations.

Geeraets, Fine and Fine (1969) reported that an opacity was observed in one eye <u>in vivo</u> which was associated with lens indentations months following CO_2 laser irradiation of the cornea. This finding suggested that delayed cataractous changes as well as lens indentation might occur.

Phillipson (1969) investigated the lenticular protein distribution in cataractous lenses produced by total eye exposure of 10 day old rats to 500 to 1,000 roentgens of x-radiation generated by a 185 kilovolt (KV) unit. He reported that the sharp gradients in protein concentration observed in the peripheral cortex of cataractous lenses closely corresponded to the interface between clear and opaque regions. He felt that the loss of transparency is related to the steep protein gradient corresponding to interfaces between regions with different refractive indices.

Kinoshita (1964) suggested that the lenticular GSH might retard the formation of intramolecular disulfide bridges. A decreased concentration of GSH might result in the formation of large insoluble lenticular protein molecules from smaller soluble proteins by means of disulfide bridges and thus might be a mechanism in some cataract formation. He, as well as Spector (1971), reported that α crystallin has a low concentration of sulfhydryl (-SH) groups in contrast to that of the β and γ crystallins.

Mach (1966) found changes in the electrophoretic pattern and associated alterations of GSH in cataractous (senile, galactose and cataracta complicata) human lenses. Her data show that the greatest decrement of both electrophoretic mobility of β and γ crystallins and GSH concentration

occurred in the senile cataracts. The electrophoretic mobility of the fastest band (α crystallin) was not decreased in any of the lens material.

Near infrared (Goldmann, 1933b), microwave (Kinoshita, Merola, Dikmak and Carpenter, 1966) and roentgen (Pirie, van Heynigen and Boag, 1953) irradiations of the eye can result in delayed lens opacities. The opacities have been observed following a delay of weeks (microwave irradiation) to months (x-radiation and near infrared). (Although Goldmann, 1933b did not measure the spectrum of his irradiation beam, it is probable from his methodology that the radiation was mainly in the near infrared.) According to Waley (1969) AsA and GSH are two reducing substances whose lenticular concentrations are reduced in mature cataracts. Kinoshita, Merola, Dikmak and Carpenter (1966) investigated the relationship of alterations of lenticular ascorbic acid and GSH to the appearance of delayed cataracts following microwave irradiation of the eyes of weanling rabbits. Lenticular opacities were not visible until approximately six days after irradiation of eyes at a wavelength of 12.3 cm with a power density of 280 mW/cm^2 for six to eight minutes. At six hours there was no alteration in lenticular ascorbic acid; at 18 hours following irradiation, the level had decreased an average of 23% compared with that of the control lenses. At this time there was no concomitant decrement of GSH in the 19 pairs of eyes assayed or in the concentration of AsA in the aqueous humor.

Pirie, van Heynigen and Boag (1953) irradiated the right eyes of weanling rabbits with 1,400 r of X radiation produced by a 200 KV unit. Occasionally the contralateral control eyes received some irradiation; it was felt that as less than 600 r of radiation was received by these eyes they could be used as controls. They measured lenticular

concentration of GSH in 15 pairs of lenses obtained at intervals in a nine month period following irradiation, AsA in a few of these lenses, and the AsA concentration in the aqueous humor of all eyes irradiated. In order to obtain sufficient material for a 20 hour post irradiation assay of GSH and AsA, they were forced to combine two pairs of lenses from the weanling rabbits. They found no changes in biochemical concentration or transparency at 20 hours in these lenses. The first change in lens transparency and GSH concentration was noted in one pair of eyes three months following irradiation. The degree of opacification increased in the remaining eyes during the ensuing six month period. This decrease in lenticular transparency was generally paralleled by a decrease in GSH concentration.

There have been a number of other investigations relating to alteration in lens metabolism associated with radiation-induced cataracts. These include investigations on albuminoid and microsomal RNA turnover, inositol concentrations, oxygen consumption and phosphate uptake, as well as mitotic activity in lenticular epithelial cells and electrolyte changes. These have been reviewed by Kuck (1970c).

It appears that only reduced glutathione (GSH) and ascorbic acid (AsA) were investigated in both x-ray and microwave cataract experiments. Furthermore, changes in GSH have been found coincident with decreased mobility of crystallins on electrophoresis of material from human cataracts (Mach, 1966). Therefore, it was felt it would be desirable to determine whether lenticular GSH and AsA were affected on infrared radiations of the eye as produced by the CO_2 wavelength. In our previous investigations

lens indentations were observed without cataract formation. This suggested that there might have been alterations of certain soluble lenticular proteins without visible coagulation. These changes might be observed on electrophoresis.

Since ulcerative perforation of the cornea following CO_2 laser irradiation could result in either an intraocular infection or permit the extrusion of the lens from the eye, it was desirable to irradiate the eye in a manner which would cause the maximal amount of delayed visible lenticular changes without corneal perforation. For these experiments one eye of each animal was irradiated with sufficient intensity to cause immediate non-perforating flattening of the cornea without the immediate whitening of the underlying lens. The other eye was used as a control.

Therefore, the purposes of this investigation were: 1) to determine any alterations of the electrophoretic patterns of soluble lens protein following irradiation of the cornea; 2) to measure any changes in lenticular AsA and GSH in these irradiated eyes; and also 3) to determine parameters which would permit non-perforating irradiations of the cornea without immediate whitening of the underlying lens. These parameters were established in the initial phase of the experiment.

Since a number of previous irradiation studies have been carried out on weanling rabbits and since changes due to irradiation might be more pronounced in such animals (van Heynigen, 1969), it was decided to carry out the investigations in this section on weanling rabbits.

Methods and Materials

Laser

The carbon dioxide laser used in this study has been described in Appendix 1. Irradiations were carried out using the multimode beam in the initial phases of the studies. In the major phase of the study, however, the laser was adjusted so that the output beam pattern produced on Thermofax paper resembled that of the TEM_{oo} mode, i.e., the power density appeared greatest at the center of the beam and decreased radially.

Beam Power Density Profile

A power density profile of the output beam was obtained by scanning the beam with moveable 3x6 cm metal plate with a centrally located 1.5 mm diameter aperture. The portion of the beam which passed through the aperture impinged on the central area of the radiant energy sensitive disc of a Coherent Radiation power detector. Then the aperture plate, attached to an x - y vernier unit was moved in 0.5 to 1.0 mm increments across the beam horizontally, then vertically. Related power outputs were plotted versus distance.

Beam Selector

A beam selector was used in order to limit the beam impinging on the eye and enable a simple pre-radiation positioning of the eye. The selector plate contained 6, 8, and 12 mm diameter apertures. The selector plate was positioned with the aid of Thermofax paper so the beam axis and an aperture center appeared to coincide. The selector plate was then mounted on a ring stand which was clamped in place in this position. The laser

was retuned for *in vivo* irradiations so the output beam was slightly larger than the beam selector aperture. The laser was further adjusted so the power distribution inferred by the pattern produced on Thermofax paper (held at the output side of the beam selector aperture) was similar to that produced by a Gaussian beam (Figure IV-2).

Animals

Albino rabbits of either sex were used in this study. Approximately 80 weanling (6-8 weeks, 1-1/2 to 2 lb.) and 12 older (up to 5 lb.) animals were used in several phases of the investigation.

Anesthesia

The animals were systemically anesthetized with a subcutaneous (s.c.) injection of 25% aqueous solution of urethane and an intramuscular (i.m.) injection of Innovar Vet (fentanyl 0.4 mg/ml and droperidol 20 mg/ml, McNeil) 1.5 and 0.15 ml respectively per pound of body weight. The pupils were dilated with an instillation of 0.5% Mydriacyl (bis-tropicamide-Alcon). The corneas were anesthetized with Ophthetic ophthalmic solution (proparacaine HCl 0.5%-Allergan) instilled 15 minutes later. When the skeletal musculature was somewhat flaccid and the corneas were unresponsive to touch (approximately 30 minutes post injection), the level of anesthesia was generally adequate for irradiation.

In Vivo Irradiations

The laser was adjusted as previously described to produce a suitable output pattern on Thermofax paper positioned immediately behind the beam selector aperture and the radiation power output was monitored with the

power detector prior to and immediately after each irradiation. The laser beam was blocked and adequately anesthetized animals were positioned behind the base selector aperture on an adjustable platform (Lab Jack). The lids were held open and an assistant checked the alignment of the cornea by sighting through the beam selector aperture; the eye was repositioned when necessary.

The exposure was initiated by unblocking the beam and simultaneously starting the electric timer by means of a foot switch. The timer (Precision Scientific Company, "Time it" P/S 69230) was graduated in 0.1 second intervals.

Preliminary Phase of the Investigation

In the initial phase of the investigation 20 weanling albino rabbits were irradiated to determine the combination of power, beam diameter and pulse duration which would result in significant biochemical and morphological lenticular alterations without causing immediate heat-induced lens whitening and minimize the potential for subsequent corneal perforations. The left eyes were irradiated and the contralateral eyes were kept as controls. The power of the irradiations was limited by the laser output and ranged from a maximum of 2 watts to a minimum of 0.8 watts. The diameter of the beam irradiating the cornea was limited by passage through 6, 8 or 12 millimeter apertures. The exposure times ranged from 20 seconds to 2 minutes.

The animals were killed either immediately post irradiation or at intervals up to 36 days post irradiation. The gross appearance of the eyes was observed, initially and on dissection. Prior to killing, the

aqueous humor was sampled from the anesthetized animals and assayed for ascorbic acid. The sampling and assay procedures are described below. Following the sampling of the aqueous humor, the animals were killed by a blow to the base of the skull or by decapitation. The lenses were dissected from the enucleated eyes and assayed for ascorbic acid and reduced glutathione. The lens removal and biochemical assay procedures are described below.

Partially on the basis of results of these irradiations and partially on the basis of the laser beam power limitations, the remainder of the irradiated animals were exposed as described below.

Major Phase of the Investigation

In the major phase of the study, the electrophoretic pattern of soluble lens protein, reduced lenticular glutathione and ascorbic acid were measured at various intervals post irradiation. To the extent possible, the ascorbic acid in the aqueous humor and oxidized glutathione in the lens were determined at several intervals following irradiation. Some ocular and rectal temperature measurements were made. A more detailed description of the methods used are given below.

The irradiations in this phase of the study were of the left eye of adequately anesthetized animals. The left eye was irradiated because of convenience in positioning the animal with respect to the laser output. These irradiations were carried out for 90 seconds with a beam with a Gaussian-like power distribution and were sufficient to flatten the cornea without obvious initial whitening of the lens. In order to minimize the apparent corneal infection observed in the initial phase of the irradiation

and prevent possible delayed corneal perforation, the animals in this phase of the study, as well as in the subsequently described ancillary experiments were treated as follows: ophthalmic Sodium Sulamyd (sodium sulfacetamide ophthalmic ointment, White) was applied to the corneas and IM injections of Crystallin G suspension (crystallin penicillin G suspension, Squibb) 10,000 units were administered immediately post irradiation and daily thereafter.

As in the primary phase of the study, the animals received 0.1 ml of Innovar Vet I.M. one and two days post irradiation to prevent gross discomfort.

Animals were killed by decapitation at the following intervals: immediately, one, two, four, five,and six days following irradiation. The eyes from these animals as well as from control unirradiated animals were enucleated and used for the electrophoretic or biochemical studies. The gross appearance of the eyes was noted. Lenses in this group were not assayed for oxidized glutathione.

Electrophoresis Experiments

Electrophoresis was carried out simultaneously on soluble lenticular protein and on aqueous humor from irradiated and contralateral control eyes. The left eyes of anesthetized animals (albino rabbit 2-5 lb.) were irradiated as discussed above; the contralateral eye served as a control. Animals were killed by decapitation either shortly after irradiation or at intervals in the ensuing four days. The eyes were immediately excised.

The lens was dissected from each eye, weighed and homogenized (room temperature) with 3 ml of Tris buffer (pH 7.4) in a glass homogenizer.

The homogenate was centrifuged at 28,000 × g for 60 minutes at a temperature of 10°C. Fluid was also obtained from the anterior chamber in certain instances. Details of the reagents, equipment and techniques employed in this and the ensuing phases of the electrophoresis are given in Appendix 2.

The anterior chamber fluid was sampled from both irradiated and contralateral control eyes of certain animals after irradiation. In order to obtain sufficient protein in the anterior chamber fluid of the control eye, paracentesis was performed on anesthetized animals at 1.5 to 2 hours prior to killing; that is, the corneas were slit and the aqueous humor expressed and discarded. The anterior chamber became refilled with "plasmoid" aqueous humor prior to killing. Following excision of such eyes, the cornea was slit again and the plasmoid aqueous humor was expressed.

Ten microliter aliquots of each lens homogenate supernatant or aqueous humor were applied to a labeled cellulose polyacetate strip which had been moistened previously with buffer (pH 8.8). The strips were aligned in the electrophoresis chamber (Gelman Instrument Company, Ann Arbor, Michigan). During electrophoresis the temperature of the strips would become elevated. Conceivably, the temperature might increase to the point at which a denaturation and coagulation of the protein samples might occur. In order to lessen this possibility, the electrophoresis unit was maintained at all times in a refrigerator at 5°C. Three hundred and fifty volts were applied across the strips for 60 minutes. The strips were then removed from the chamber, fixed and stained by immersion in Ponceau S solution. The stained bands were

counted and the respective distances between each and the site of original sample application was measured. The distance was a function of the mobility of each group of soluble proteins. The mobilities of bands of lenticular proteins from irradiated eyes were compared with those of from the contralateral control eyes.

Ascorbic Acid and Reduced Glutathione Determinations

Ascorbic acid (AsA) was assayed by the method of Roe (1954). This was carried out in either aqueous humor or lens material from pairs of eyes from the same animal. Sampling procedures and preparations of aqueous humor and lenses for this and the reduced glutathione (GSH) assays are given below. The preparation of standard curves and assay procedures for ascorbic acid are listed in Appendix 3. The concentration of AsA was expressed as mg% (milligrams of AsA per 100 ml of aqueous humor or mg per 100 grams of wet weight lens). The concentration of AsA in material from irradiated eyes was expressed as a percentage of that in the contralateral control eye.

Reduced glutathione (GSH) was assayed by a modified method of Grunert and Phillips (1951). These assays were carried out concurrently on pairs of lens from both unirradiated and irradiated animals. The preparation of standard curves and assay procedures are listed in Appendix 4. The lenticular concentrations of GSH were compared; following irradiation the concentration of the GSH in the lens from each irradiated eye was expressed as a percentage of that in the contralateral control lens.

Sampling of Aqueous Humor and Lenses

Aqueous humor was sampled from control eyes of anesthetized animals with a heparinized 50 uL Hamilton syringe (heparin solution 10 mg/ml had been previously drawn up into the syringe and expelled from the barrel and needle). The lessened transparency of the irradiated corneas did not always permit the proper insertion of the sampling needle into the anterior chamber. Furthermore, even heparinized syringes became blocked during sampling attempts. Therefore this method was abandoned.

In order to sample aqueous humor from irradiated eyes, the following alternate procedure was followed: the animal was decapitated, the eyes were enucleated, rinsed in normal saline and blotted dry, and placed in labeled dry plastic vials that were immersed in crushed ice. The corneas were slit and the aqueous humor expressed on a labeled tared Parafilm square. The weight of the aqueous humor was determined by difference.

In order to assay the aqueous humor for ascorbic acid, either the measured volume from the syringe (using the previous method) or the weighed volume was admixed with 2 ml of 5% trichloracetic acid (TCA) and was centrifuged at 5° C at approximately 12,000 x g (desk centrifuge operated in a laboratory refrigerator). The TCA supernatant was assayed for ascorbic acid.

The lenses were removed from enucleated eyes after the zonules were cut; the adhering vitreous body was cut from the posterior surface of the lens. The lens was blotted free of adhering droplets on a section of filter paper previously moistened with normal saline, placed in labeled

tared 7 ml tissue grinders and weighed. The lenses were then homogenized in 3 ml of ice-cold 10% TCA. The homogenate was centrifuged (approximately 12,000 x g for 10 minutes at 5° C). The supernatant was filtered through Whatman No. 1 filter paper cut to fit a buret funnel.

Ancillary Experiments

The following short investigations were carried out in the hope that they might assist in the interpretation of certain data obtained in the major portion of this investigation. First, temperature measurements were taken in an attempt to understand the mechanism behind the occasional appearance of immediate lens whitening on irradiation. Second, lenticular GSH and GSSG were concurrently determined following either irradiation of the eye or mechanical trauma to the lens. This was done in an attempt to understand the mechanism of the decrement of lenticular GSH observed following irradiation.

Although the power and duration of the exposures in the major phase of the investigation had been chosen to cause maximal damage without immediate whitening of the lens, a small percentage of lenses could not be used for the electrophoretic or biochemical studies because they had been whitened. This apparently resulted from heat. Therefore, it was of interest to measure the maximum *in vivo* temperature in the weanling rabbit eye during irradiation of the cornea. Attempts were made to determine the temperature of the aqueous humor by inserting a thermocouple into the anterior chamber as described in Chapter II. Although this technique had been employed successfully in the measurements during irradiation of the larger eyes of mature rabbits, it could not readily be used in the small

eyes of weanling rabbits. Therefore, an alternate procedure was employed. The weanling rabbits were irradiated as described and the corneal surface temperature was measured with the flat-ended thermocouple as described in Chapter II. At the end of the 90 second irradiation a slit was made through the center of the corneas into the anterior chamber. The temperature of the extruded aqueous humor was measured immediately with the thermocouple. The maximal temperature of an eye during irradiation is dependent not only on the temperature elevation, but also on the initial pre-irradiation temperature of the eye. That is, the maximal temperature attained for each temperature elevation would vary as the initial temperature varied. This was reported by Goldmann (1933a) who also showed that the decrements of rectal temperature observed on the administration of anesthetic agents is reflected by a decreased temperature in the anterior segment of the eye. Thus, the pre-irradiation ocular temperature could have been an inverse function of the length of time between the administration of the anesthetic agents and the initiation of the irradiation. In order to determine if the co-administration of these two drugs might have had a marked effect on the body temperature, measurements were made of the rectal temperature of rabbits at intervals in the 30 to 45 minutes following the parenteral administration of Urethane 25% 1.5 ml/Kg and Innovar Vet 0.15 ml/Kg as described above. The rectal measurements were made with a mercury thermometer (Wills Scientific, 1 to 55 C, calibration checked by immersion in a mixture of ice and water). The thermometer was inserted to a depth of 45 mm at 5 to 10 minute intervals after the administration of the drugs and the temperatures recorded.

The third phase of the ancillary experiments was carried out to assist in the interpretation of the decrement of GSH in the lenses from irradiated eyes. Two causes of decreased lenticular GSH could be either a decreased synthesis of the tripeptide within the lens, or an increased oxidative conversion of reduced glutathione to oxidized glutathione (GSH to GSSG). (The latter situation should be associated with a lessened ratio of GSH to GSSG, which is normally approximately 10:1, Kuck, 1970b.)

Concurrent assays for lenticular GSH and GSSG were carried out. GSH was measured by the method of Grunert and Phillips (1951) as described in Appendix 4, and GSSG was measured by a modified method of Bergmever (1963) as described in Appendix 5. Larger (5 lb.) animals were used to provide more lens material for these assays.

For comparison, in one study two rabbits were irradiated (1.5 watts for 90 seconds) and killed five days later. In a second study, the anterior lens capsule in one eye of each of two animals was torn.

The procedure was carried out as follows: The animals were anesthetized as described above, the eyes were proptosed to facilitate needling done with a sterile microdissection needle. In one animal (N-1) the needle was held horizontally and thrust through the cornea immediately anterior to the limbus. The tip of the needle was then inserted into the anterior cortex of the lens, then moved so that a 4-5 mm tear was made in the capsule and underlying lenticular fibers. The left eye of the second animal (N-2) was punctured by thrusting the needle along the axis of the eye through both the cornea and anterior capsule of the lens into the subjacent lenticular fibers. The animals were treated with penicillin and sodium sulamyd

ophthalmic ointment as described above. The animals were killed five days later and the GSH and GSSG assayed in the lenses. This second study was carried out in an attempt to observe the effects of oxidation on the lenticular ratio of GSH:GSSG. Prince and Eglitis (1964b) cite a report that rupture of the capsule of the lens increased the uptake of oxygen by the lens by over 400%.

Results

Irradiation Beam Characteristics

Figure IV-1 shows the pattern produced on Thermofax paper by the laser operating in a multimode condition. On short exposures the darkening is an approximate indication of the power density. The variation in pattern density indicates zonal differences in the beam power density distribution.

Figure IV-2 shows the pattern produced on Thermofax paper by a beam assumed to be operating in a TEMoo mode (Transverse Electromagnetic Mode in which the power density has a Gaussian distribution).

In Figure IV-3 the relative power density is plotted as a function of the radial distance across the beam. The power density is seen to vary inversely with the distance from the center of the beam. The power density distribution measured was that produced by a beam similar to that causing the pattern observed in Figure IV-2.

In Figure IV-4 is shown the pattern produced on Thermofax paper by the expanded laser beam after passage through an 8 millimeter diameter aperture. The pattern of darkening was similar to the pattern of Figure IV-2. Except for the preliminary irradiation studies, the irradiations

in this chapter were carried out with a beam which gave this pattern on Thermofax paper.

Biological Studies

Anesthesia

Thirty minutes after the injection of the anesthetic agents, the skeletal musculature was somewhat flaccid. The corneal reflex was absent or minimal and the pupils were apparently fully dilated (5-6 mm vertical diameter). In general, there was no obvious respiratory distress noted in the anesthetized animals. The level of anesthesia obtained with this regimen (Urethane 25%, Innovar Vet, 1.5 and 0.15 ml respectively per pound of body weight) resembled that of Stage III (Vandam, 1958) and was adequate to permit corneal irradiation. Using this regimen, less than 10% of the anesthetized animals died spontaneously either during or immediately following irradiation.

In Vivo Irradiations

Preliminary Phase of the Investigation

During preliminary irradiation studies, purulent keratitis appeared to develop in all eyes irradiated at power densities greater than 2 W/cm^2 for longer than 20 seconds. Examination of these eyes two or three weeks post irradiation showed that the corneas had perforated. Dissection and gross examination of the interior of the globe revealed one of the following: 1. An amorphous mass of purulent material; 2. a fibrous structure consisting of a flat round plate in the lenticular region connected by a thin stalk to the optic disc and the absence of a recognizable retina;

and 3. a grossly normal appearance except for the absence of a lens.

Irradiation at lower power densities for less than 10 seconds apparently affected only the cornea. The corneas were scarred and whitened in the irradiated areas two to three weeks post irradiation. The degree of corneal damage appeared to have been proportional to the irradiation power density and duration. Upon dissection and gross examination, the interior of these eyes appeared normal.

Major Phase of the Investigations

Gross appearances

These eyes were irradiated at 1.5 watt, 0.8 cm diameter, 90 second exposure followed by daily local and systemic treatment for purulent keratitis.

After the initial 12 to 15 seconds of irradiation, the corneas became noticeably hazy; as the exposure continued the clouding increased. After 30 seconds of exposure the corneal surface was pitted. By the end of the 90 second irradiation the corneal surface had flattened; at this time the corneas appeared to be in contact with the iris and lens surface. Pre-irradiation, the rabbit pupils were generally circular; however, some appeared to be slightly ellipsoidal, with the major axis in the vertical direction. Post irradiation the pupils looked similar, but smaller. The vertical pupil diameter had decreased from 5 to 6 millimeters pre-irradiation width to 4 millimeters; the horizontal diameter decreased correspondingly.

Immediately post irradiation the cornea was cloudy, but this did not preclude visualization of either the iris or the pupillary region of the

lens. A small percentage of the lenses were obviously whitened at this time; these eyes were not used for electrophoretic or biochemical determinations. In most instances the superior portion of the iris was blanched; the vasculature on the remainder of the anterior surface of the iris was noticeably distended.

Gross dissection of eyes immediately post irradiation showed that the corneas were in close apposition to the irides and anterior lens surfaces. Removal of the iris revealed that the tinctorial pattern on the posterior surface was grossly similar to that observed on the anterior surface, i.e., there were similar regions of blanched and distended vessels.

Certain eyes were enucleated, incised and fixed in glutaraldehyde immediately following irradiation. Complete iridectomies of these fixed eyes performed following removal of the corneas exposed the remainder of the anterior lens surface. Occasionally, this revealed the imprint of the posterior iridial processes in the superior pre-equatorial region of the lens. The zonules at the superior equatorial region of the lenses were often whitened or broken; occasionally the lens capsules were torn along the equator in this region.

Ninety minutes after irradiation, the corneal curvature appeared to have returned to normalcy. Some of the corneas developed a bluish hue by this time.

One day post irradiation the corneas were cloudier and bluer than they had been immediately post irradiation; whitish material present on the surface of the eye appeared to have sloughed from the corneal surface. The majority of the irides were still blanched in the superior region and

showed distended vasculature, particularly in the inferior region. Gross dissection of these eyes showed the appearance of a thin subcapsular layer of liquid in the superior pre-equatorial regions of the lenses. In some instances, this liquid scattered light; expression of the material through a capsular puncture wound restored optical clarity to this region. In a few instances, a thin white arcuate line was observed on the lens immediately inferior to the area of subcapsular liquefied material.

Two to three days following irradiation the external appearance of the exposed eyes had not altered remarkably. White flocculent material continued to slough from the corneal surface. Four to five days post irradiation the central cornea had sloughed off exposing a somewhat transparent tissue. Gross dissection of these eyes suggested that the clear tissue was Descemet's membrane. There was little aqueous humor in the majority of the eyes; often there was concomitant blanching of the entire iris. The iris appeared to be compressed between cornea and lens. The zonules were whitened and broken, especially along the superior half of the lens equator. The lens appeared to be displaced anteriorly. The anterior lens surface was hazy and an occasional white arcuate region was present on the anterior pre-equatorial surface of some lenses. The superior equatorial cortex was liquefied in a majority of the lenses examined at this time. Following fixation of these lenses in glutaraldehyde, the firmness of the liquefied region could not be distinguished from the remainder of the lens. Many lenses were spontaneously decapsulated during excision, probably because of a tear in the equatorial capsule. Whitened vertical lines were observed on the superior half of the posterior of a majority of lenses examined at this time.

Five days post irradiation some of the corneas were conically distended. On dissection the lenses had visibly deteriorated; the volume of liquefied material had increased and a larger area of the posterior surface was whitened. Six days post irradiation the lens volume had markedly decreased and the remaining lens material was whitened.

Electrophoresis Investigations

Simultaneous electrophoretic separation of lenticular proteins from pairs of lenses from normal eyes always resulted in patterns which were identical in the number of clearly visible bands and in their mobilities. Although the electrophoretic pattern was identical between pairs of normal lenses from the same animal, it was not identical when the protein was taken from lenses of different rabbits. For example, in many instances, one could readily see six bands identifiable as α, β, and σ crystallins (based on Mehta and Maisel, 1966); following other separations only five clearly distinguishable bands could be seen. Furthermore, occasionally a narrow fast band could be seen (faster than the α crystallin band).

Figure IV-5 shows the electrophoretic pattern of soluble lenticular proteins from unirradiated rabbit eyes. The pattern on one strip represents the soluble proteins from one lens; the identical pattern on the other strip are from the contralateral lens. The six bands (I to VI) traveled different distances from the point of application (0). The relatively wide band with the greatest mobility (I) is probably α crystallin; the bands of intermediate mobility (II and III) are probably β crystallin; and the slowest bands (IV, V, and VI) are probably γ crystallin (data from Mehta and Maisel, 1966, served as the basis for this correlation of crystallin and electrophoretic bands).

Figure IV-6 shows the electrophoretic patterns produced by the lens proteins taken from an eye immediately following irradiation and compared with that of the contralateral control lens. There are six bands on each strip which appear identical in mobilities and widths.

Figure IV-7 shows the separation of lens material taken three days following irradiation. In contrast to the previous figures, the mobilities of the bands are not identical between proteins from lens of irradiated and contralateral control eye. Five bands are visible on the strip containing lens material from the exposed eye (L). The same bands are visible on the strip containing material from the control lens (C), plus an additional fast band (La). This fast band was occasionally visible following electrophoretic separation of lenses from either irradiated or control eyes, and appeared ahead of the fast α crystallin (I) band. The bands from the exposed lens appear to have traveled a shorter distance than those from the contralateral control lens. Expressed as a percentage of the corresponding bands in the control lens strip, the mobilities were: I, 92%; II, 81%; III, 79%; IV, 75%; and V, 83%. These values were based on measurements made immediately following the electrophoretic separation and may be slightly different from those on the dry strip in the figure.

Comparison of the mobilities of lenticular protein made four days following irradiation may be made from Figure IV-8. The mobilities expressed as percentages of the corresponding band from the control lens (C) were: I, 89%; II, 61%; III, 58%; IV, 45%; and V, 35%. Although all the bands from the irradiated lenses had lessened mobilities at this time following irradiation, the mobility of band I (α crystallin) was only slightly depressed, whereas, that of the remaining bands (II to V), the β and σ

crystallins, were quite slowed. The two remaining strips in Figure IV-8 shows the pattern produced by liquid from the anterior chambers of the irradiated and control eyes. The strip containing the plasmoid aqueous humor from the control eye shows six bands. The fastest and widest band is probably serum albumin (this is inferred from data of Moore, 1959). The fast albumin band (Alb) had a much greater mobility than the fastest lenticular protein Band (Band I). In fact the slowest visible band of the plasmoid aqueous humor proteins had a mobility nearly equal to the I band of the lenticular protein.

The pattern produced by the aqueous humor from the irradiated eye was quite different from that of the control eye. The fast Alb band was still quite visible; however, the pattern appeared to contain the I band of the normal lens protein, both in mobility and band width. Also, the slowest IV and V lens protein bands seemed present; again the band width and mobility equalled that of the normal lens. The pattern produced by this aqueous humor appeared to be a composite of plasma and lenticular proteins. It is not surprising that lenticular proteins were present in the aqueous humor of the irradiated eye as the lens capsule had spontaneously separated from the remainder of the lens during dissection and the lens weight was approximately 70% of the contralateral values

Ascorbic Acid and Reduced Glutathione Determinations

<u>Ascorbic Acid Measurements of Aqueous Humor</u>

When sampled with the Hamilton microliter syringe, the average concentration of ascorbic acid (AsA) from control eyes was 35 mg%, ranging from 25 to 43 mg%. The concentration of AsA in pairs of unirradiated eyes from the same animal agreed within 4%.

Sampling of the anterior chamber fluid from irradiated eyes with the syringe was extremely difficult because proper placement of the needle tip in the anterior chamber of the irradiated eyes could not be readily accomplished on account of the lessened transparency of the corneas. As one did not have a clear view of the path of the inserted needle tip, it could be inserted inadvertently into iridial or lenticular tissue. Furthermore, it was not possible to withdraw sufficient material for assay since the plasmoid aqueous humor clotted and occluded the needle lumen. Therefore, aqueous humor of irradiated eyes could not be obtained by direct sampling for AsA determinations. Consequently, for comparison of AsA in aqueous humor in control and irradiated eyes, the eyes were excised and the anterior chamber fluid expressed through a slit made through the center of the cornea into the anterior chamber.

With this method, the aqueous humor AsA concentration in pairs of unirradiated as well as irradiated and contralateral control eyes was measured (Table IV-1). The average concentration of AsA in aqueous humor expressed from unirradiated eyes was 17.4 mg% and ranged from 9.4 to 31.3 mg%. These values from pairs of control eyes showed AsA concentration ranging from 81 to 124 percent of the contralateral value (Table IV-1). Agreement from pairs of control eye aqueous humor AsA was not as good as with the syringe technique.

The ascorbic acid concentrations of the anterior chamber fluid from irradiated eyes always decreased when compared with that of the contralateral control eye, using the corneal slit method. Two hours post irradiation the concentration in the exposed eye could only be determined for one

pair of eyes because of difficulty in obtaining adequate liquid. The value for the AsA concentration in this eye was 39 percent of the contralateral control eye value. One day post irradiation the value was 54 percent. Adequate material was not obtainable from the three day post irradiation group. Five days post irradiation the value was 33 percent. Six days post irradiation adequate material could not be obtained for assay. The decrement of AsA concentration of the aqueous humor of irradiated eyes appeared to have occurred previous to that in the lenses from the irradiated eyes.

Lens Investigations

Lens weights

The average control lens weight was 0.212 grams; the weights ranged from 0.1750 to 0.269 grams (Table IV-1). The weights of pairs of lenses agreed within 17 percent.

Figure IV-9 is a plot of the average lens weight from irradiated eyes expressed as a percentage of the contralateral control lens as a function of time following irradiation.

Initially, following irradiation, the lens weights were rather constant and not greatly different from the contralateral control lens weights. Two hours post irradiation the average percentage relative to that of the contralateral control lens weight was 82 percent; one day after irradiation 104 percent; three days, 89 percent, and 5 days, 89 percent.

Lenticular ascorbic acid

The average value of ascorbic acid (AsA) in the control lenses was 14.1 mg%; the values ranged from 8.2 to 18.8 mg% (including pairs of

unirradiated lenses). The concentrations of AsA in pairs of lenses from unirradiated eyes (Table IV-1) were compared and the concentrations agreed within 8 percent.

The concentration of AsA in each irradiated lens was expressed as a percentage of the concentration in the contralateral control lens (PCL). The average of these values in each irradiation time group were plotted versus time after irradiation (Figure IV-10). The ordinate is graduated in percent, the abscissa, in days post irradiation. The curve appeared to be somewhat parabolic in shape. Two hours after irradiation the percentage for lens ascorbic acid was 87 percent (average); one day after irradiation, 95 percent; three days after irradiation, 54 percent; and five days after irradiation only 34 percent. Six days post irradiation the value had decreased to approximately 23 percent; it was barely measurable.

Lenticular reduced glutathione

The concentration of reduced glutathione (GSH) in each lens from an irradiated eye was expressed as a percentage of the concentration in the contralateral control lens (Table IV-1). The values in each post irradiation time group were averaged. In Figure IV-11 these values are plotted versus time after irradiation. The resultant curve appears grossly similar to the plot for lenticular ascorbic acid concentration described above.

Two hours after irradiation the average percentage of the contralateral control lens concentration was 93%; one day post irradiation, 99%; three days post irradiation, 47%; five days post irradiation, 40%, and

six days post irradiation was zero.

During the first three days p-radiation the lens weight remained constant; the AsA and GSH concentrations had started to decrease three days following irradiation. Four to five days post irradiation the AsA and GSH concentrations continued to decrease. Lens changes were noticeable at this time; increased volume of cortical liquefaction, whitened lines on the posterior surface and spontaneous decapsulation on excision occurred. The nearly total disappearance of lenticular AsA and GSH observed six days post irradiation was accompanied by a marked decrease of lens weight. The remaining lens material was nearly completely white. These data (concentration and weight) suggest that biochemical changes may occur previous to changes in lens weight. The concentrations of AsA and GSH were expressed as a percentage of wet weight. Normally, the young rabbit lens contains approximately 75% water (Prince, 1964). It is possible that the water content changed in the period during which these measurements were made.

In Figure IV-12 the total weight of glutathione and ascorbic acid per lens (concentration times weight) were also expressed as a percentage of the contralateral (control) lens GSH or AsA weight per lens (Av PCL Wt). These values were averaged for each irradiation group for GSH and for AsA. Figure IV-12 is a plot of these values as a function of time post irradiation. The ordinate is graduated in percent; the abscissa, in days post irradiation. The two curves nearly paralleled one another; however, the decrement of AsA per lens was slightly greater than that of the corresponding total values for glutathione per lens following irradiation.

Two hours following irradiation the Av PCL Wt for AsA was 73%; that of GSH was 80%. One day following irradiation the Av PCL Wt value for AsA was approximately 99%; that of GSH was 103%. Three days following irradiation the Av PCL Wt value for AsA was 45%; that of GSH was 43%. Five days post irradiation the Av PCL Wt value for AsA was approximately 30%; that for GSH approximately 35%. Six days post irradiation the Av PCL Wt value for AsA was approximately 1%. No measurable amount of GSH was found at this time.

Ancillary Investigations

During irradiation the corneal surface temperature increased from an average of 31.6° C to 61.6° C (an average increase of 30°C). The average maximal temperature of 44° C was measured in the aqueous humor extruded through a slit made in the cornea immediately following the cessation of irradiation.

The decrement of rectal temperature measured following the administration of urethane and Innovar Vet are shown in Figure IV-13. The temperature decreased nearly linearly from an approximate value of 40° C. The average rate of decrease following the drug administration was approximately 1.3° C/30 minutes in these two measurements. Although the corresponding anterior chamber temperature changes were not measured in this experiment, Goldmann (1933a) reported that the temperature would decrease correspondingly.

The results of concurrent assays of lenticular reduced glutathione (GSH) and oxidized glutathione (GSSG) in animals following either CO_2 laser irradiation of the cornea or needling of the lens were as follows:

Lenses were dissected from eyes excised six days following irradiation of two 5-lb animals. Lens L-1 consisted of an amorphous mass of flocculent white material. The cortical material of the superior equatorial and posterior region of lens L-2 was whitened; the remainder of the lens was relatively clear. Two 5-lb rabbits were killed five days following needling of the left eyes. Lens N-1 had an obviously torn anterior lens capsule and a whitened cortex. The other needled lens, N-2, was grossly normal except for a small white puncture spot in the mid anterior surface of the lens. Values for these lenses are expressed as percentages of that in the contralateral control eye (except for the values of GSSG as percentage of total lens glutathione (GSSG+GSH)). This value was computed for each lens.

Lens No.	Weight	GSSG Concentration	Weight	GSH Concentration	Weight	$\frac{\text{GSSG}}{\text{GSSG+GSH}} \times 100$
L-1	51%	49%	25%	58%	30%	4.5%
L-2	78%	66%	52%	75%	58%	4.8%
N-1	75%	139%	100%	66%	59%	16.0%
N-2	100%	112%	113%	87%	89%	10.0%
Control lenses (lasered animals)						5.0%
Control lenses (needled animals)						8.0%

It appears that both oxidized and reduced glutathione had decreased in concentration and in weight in lenses from irradiated eyes. The relationship of GSSG to total glutathione, however, had not changed from that in the contralateral control lenses. In contrast, data from the needled lenses indicate that although the GSH had decreased, the GSSG had

correspondingly increased. In fact, the percentage decrease of GSH is almost exactly offset by a corresponding increase in the percentage of GSSG concentration. Furthermore, the amount of GSSG had increased noticeably—the ratio $\frac{GSSG}{GSSG+GSH}$ of the more severely injured lens doubled.

Discussion

Beam Adjustment and Measurements

For these studies it might have been desirable to obtain uniform corneal irradiations. With the laser used, however, it was difficult to obtain and maintain a beam pattern which was essentially uniform over the irradiated diameter desired at the power density levels required. Consequently, the central part of an essentially Gaussian beam was used during these irradiations..

Thermofax paper is darkened by heat; low CO_2 laser output densities (approximately 50 mW/cm^2. are not adequate to darken the paper, even on long exposures (minutes); high power densities (greater than 50 W/cm^2) nearly simultaneously blacken and ignite the irradiated area. On protracted intermediate CO_2 power density exposures heat flows through the periphery of the irradiated region resulting in a pattern larger than the impinging beam. Short exposure of intermediate power, however, results in a pattern whose varying degrees of darkness is roughly a function of the local power densities. The laser was, therefore, adjusted by observing the output pattern produced on Thermofax paper on short exposure.

It was felt that it would be impractical to carry out a beam scan for each irradiation. To substantiate the assumption that the darkened pattern

used as a monitor during these experiments would be satisfactory, a beam profile was measured for such a pattern (Figure IV-3). The Gaussian-like profile obtained in the X and Y directions was such that it was felt that monitoring with the Thermofax paper would be satisfactory during these experiments.

There may have been slight inaccuracies in the power density profile values measures (Figure IV-3) due to inequalities of the response of the radiant energy beam detector to different power densities, as the aperture was scanned across the detector. The measured power density profile might have been more accurate if the power detector, fitted with an aperture, had been scanned across the beam; however, the weight and bulk of the power detector precluded such an operation. The diameter of the beam was increased for in vivo irradiations in order that the greater portion of the corneal surface would be exposed. The laser was retuned so the beam diameter was slightly larger than, and thus limited by the beam selector aperture. The power density distribution (as inferred by the pattern produced on Thermofax paper) (Figure IV-2) appeared similar to the pattern on paper associated with the beam pattern described above. The power density distribution of this larger beam was not measured because of the relative insensitivity of the available power measuring equipment.

Use of the beam selector aperture permitted simple location of the beam axis and furthermore defined the beam irradiating the eye. When the laser was adjusted so the output beam pattern on Thermofax paper (held approximately 0.8 cm distal to the beam selector aperture) was concentric with and filled the aperture, one could then irradiate the entire corneal

surface with minimal exposure of the ocular adnexa. Furthermore, when the axis of the eye and that of the laser beam coincided, the maximum power density of the beam and thus the maximum heating coincided with the center of the cornea. When the cornea flattened, the temperature of the underlying aqueous humor in the pupillary region was probably highest at the center. Therefore, when the cornea and lens were apposed following corneal flattening, the aqueous humor in the pupillary region (exposed lens region) may have been heated to a greater temperature than that of any other region of the anterior chamber. The maximum heating and thus the initial collagen shrinkage occurred at the center of the cornea. There may have been other regions of temperature differences. As discussed below, the temperature in the superior portion of the anterior chamber may have been greater than that of the inferior region.

Anesthesia

Innovar Vet is a combination of a synthetic morphine-like compound (fentanyl) and a tranquilizer (droperidol). High doses of either of these class of compounds, especially the morphine-like substance, can depress the respiratory center (Sharpless, 1965). Pentobarbital, a medium length duration type barbiturate, will also depress the respiratory center when employed in adequate doses.

In a previous study Innovar Vet (I.M.) and pentobarbital (I.V.) per se or in combination were employed as anesthetic agents. The use of Innovar Vet resulted in an apparent increase of fluid in the respiratory tract and resultant distress. Atropine sulfate (S.C.) was used to depress the fluid production. Anesthetic doses of pentobarbital apparently did

not cause respiratory distress from fluid accumulation in the lungs. Such doses, however, often caused death, seemingly due to respiratory depression.

The respiratory distress accompanying adequate levels of anesthesia was greater when Innovar Vet was used, than when pentobarbital was used (both were used in conjunction with atropine sulfate). In the previous study, more than 25 percent of the rabbits died during or shortly after irradiations when anesthetized using either or both of these substances, alone or in combination. Furthermore, horizontal nystagmus was often present in animals thus anesthetized. This movement of the eyes during irradiation often prevented protracted even irradiation of selected areas of the cornea.

Accordingly to Shideman (1958) urethane (ethyl carbamate) has been used for many years as an anesthetic agent in animal experiments. Massive doses of urethane can produce protracted unconsciousness and anesthesia without fatal respiratory depression. In this study relatively safe and adequate levels of anesthesia were obtained in weanling albino rabbits with the systemic administration of urethane (S.C.) and Innovar Vet (I.M.) (1.5 and 0.15 ml respectively per pound of body weight) plus the topical administration (to the eyes) of proparacaine HCl ophthalmic solution. When the animals were adequately anesthetized, there was no noticeable respiratory distress and no observable nystagmus. The lack of nystagmus permitted continuous even irradiation of the cornea.

The local anesthetic proparacaine was originally used to permit the painless insertion of a speculum. Although a speculum was not employed

in this study, the use of proparacaine was continued. The animals systemically anesthetized with urethane and Innovar Vet could not be irradiated without some resistance unless either the quantity of the systemic anesthetic agents was increased or proparacaine used.

Less than 10 percent of all animals anesthetized in this manner died spontaneously during or following irradiation. This method of anesthetizing weanling rabbits appeared to be superior to those previously employed in our studies.

The use of 0.1 ml of Innovar Vet, I.M. one and two days following the irradiation may not have been necessary but was routinely adminstered to avert gross discomfort.

In Vivo Irradiations

Preliminary Phase of the Investigation

The effect of the post irradiation corneal perforation appeared to differ. In certain of our preliminary irradiation experiments, the lens was absent on opening the eye. The lens may have been extruded through a corneal perforation. Serial sections of the cornea would clarify this. As the animals were housed in standard cages following irradiation, this could have occurred without being noted.

It is also possible that resorption of the lens cortex and nucleus may have occurred. This may have been precipitated by cornea-lens contact following loss of anterior chamber aqueous humor via a perforated corneal ulcer. Also the lens capsule may have been ruptured during or following corneal irradiation. Subsequently, the exposed cortical material could have been altered on contact with aqueous humor, then resorbed by diffusion.

Resorption could have also resulted from an immune process. A combination of these processes may have been responsible for resorption of lens material.

It has been reported clinically that both lenses and vitreous humor have been resorbed following injury and subsequent infection. According to Duke-Elder (1970), the resultant gross appearance of such an eye externally is one of microphthalmia, and internally, of complete retinal detachment. The retina remains attached at both the ora serrata and the optic nerve. This description is essentially identical to that of certain irradiated eyes examined in this study following enucleation and fixation several weeks after irradiation. The interior of the microphthalmic eyes contained a fibrous plaque in the lens region attached by a thin fibrous stalk to the optic nerve. Apparently, following retinal detachment (and subsequent fixation) the opposed surfaces contacted and adhered.

The purulent mass observed filling other eyes probably was the end result of an overwhelming panophthalmitis caused by infecting organisms which entered the eye through the perforated corneal ulcer.

Major Phase of the Investigation

The concomitant administration of topical sodium sulfacetamide and systemic penicillin, following 1.5 watt 90 second corneal irradiations prevented the purulent keratitis. Although the offending organisms were not isolated and identified, this treatment regime proved to be efficacious. The simultaneous administration of a sulfonamide and an antibiotic is an effective and not uncommon treatment (Weinstein, 1965) which is simple and readily available. Sodium sulfacetamide is reportedly non-

irritating to the cornea and penetrates readily into corneal tissues (Havener, 1970). The following is a discussion of the gross appearance of the corneas, irides and lenses following irradiation.

Gross Appearance

The pitting of the corneas and the attendant meiosis observed following irradiation have been discussed in Chapter II. The appearance of the corneas immediately following irradiation differed from that observed in the ensuing day; that is, the corneas became whiter and developed an ever increasing blue hue with time. This blue hue was first observed one to two hours post irradiation. The initial whitening post irradiation was probably due to light scattered from coagulated corneal protein.

The increased corneal thickness associated with edema observed may have been partially responsible for the increased whitening and blueness observed with time. With edema, the transparency decreases and scattering increases. According to Maurice (1969) only a small amount of light is scattered by the normal cornea. Scattering is greater at shorter than at longer wavelengths. Maurice shone a beam of white light along the axis of a strip of excised cornea. The tissue near the entry of the beam glowed blue and that remote from it glowed red. (Maurice postulated that the transparency of the normal cornea is dependent on the presence of a regular crystalline-like arrangement of the collagen fibrils. The interfibrillar spacing is said to become irregular in the edematous cornea.) Alternately, there may have been additional denaturation of proteins occurring with time, thus increasing the whitening and blueness. A combination of

the above two mechanisms may have been responsible for these changes in the appearance of the corneas following irradiation.

Although histopathologic examination was not carried out, the white flocculent material observed floating on the corneal surfaces two to three days post irradiation probably was clumps of necrotic epithelial and stromal cells.

The clear central region of the cornea observed about four days following irradiation probably was Descemet's membrane. Apparently the necrotic whitened stromal material had sloughed off completely from this region. Descemet's membrane is the basement membrane of the endothelial cells of the cornea (Fine and Yanoff, 1972). This layer must have been subjected to a temperature elevation similar to that of the adjacent corneal stroma during irradiation of the cornea. Nevertheless, its structure appeared to have been affected to a lesser degree than that of the corneal stroma, as it remained following sloughing of the outer layers.

The conical distention of the corneas along the optic axis observed five to six days post irradiation suggests that the corneal tissues which remained following erosion of the anterior layers were weaker than the intact cornea. They may have been distended by either normal or increased intraocular pressure.

There are several factors which may have been responsible for producing the adjacent areas of blanching and normal color in the irides of the irradiated eyes observed following irradiation. The blanching of the superior portion suggests that the temperature of this region may have been increased to a greater level than the remainder of the anterior chamber during CO_2 laser irradiation of the cornea. Temperature measurement in

this investigation indicated that the temperature of the aqueous humor present in the pupillary region of the anterior chamber at the termination of the 1.5 W, 90 second irradiation had been elevated to at least 44° C. Probably the temperature elevation in the anterior chamber was considerably higher. (Steady-state temperature data from Chapter II show that on continous irradiation of the corneas of larger rabbit eyes at a power density of 380 mW/cm^2, the aqueous humor temperature was elevated to 48° C without concomitant blanching of the irides. The corneas of these larger animals, however, were not flattened during these lower power density irradiations.)

Possibly, during irradiation the heated aqueous humor rose to the superior region of the anterior chamber and increased the temperature of this portion of the cornea and irides to a higher level than that of the remainder of the tissue. Because of the higher temperature, maximal edematous swelling of the cornea may have occurred in this region. This would have decreased the depth of the anterior chamber in the superior region to a greater extent than the remainder of the chamber. During corneal flattening either the pressure of the cornea on the iris or the increased pressure in the anterior chamber may have squeezed the blood from the two superior branches of the major iridial circle. (According to Prince and Eglitis, 1956a, the major iridial circle is supplied with arterial blood from two main branches, one enters nasally, the other temporally. After entering the iris, each bifurcates and sends branches superiorly and inferiorly.) If the blood were forced from the superior branches during flattening of the cornea, their counterparts in the inferior region may have been temporarily engorged. Once the blood flow

was stopped in the superior portion of the irides, a major portion of the mechanism for the cooling of this region was removed. Heat from the cornea as well as a limited flow of heated aqueous humor to this region may have caused a marked increase of the temperature of the superior region with resultant permanent local damage to the vasculature. Although histology was not carried out in this investigation, Hoffmann and Kunz (1934) published descriptions of the histology of heat-damaged iridial tissue. They noted that the iridial arteries were generally empty of blood and that many of the capillaries in the deeper regions of the irides were alternately constricted, so the appearance was that of links of sausages. Their findings have been reviewed in Chapter II.

Davson (1969) stated that the active production of aqueous humor is dependent on the associated blood flow in the ciliary processes. Therefore, on the basis of the loss of circulation in these eyes, at least one-third of the total ciliary process secretory mechanism may have been halted.

The blood flow did not return to the blanched superior third of the irides following irradiation. This indicated that the mechanism which caused the occlusion during irradiation was permanent or had been replaced by an alternate mechanism. It seems that the arterial supply must have been occluded initially. If the venous system had been closed, the blood flow would have been halted; however, the tissue would have contained trapped bloow, and thus would not have been blanched. Apparently, the vascular occlusion was caused by heating, perhaps in conjunction with pressure elevation.

The blanching of the entire iridial and ciliary processes observed five to six days post irradiation indicated a complete stoppage of the

associated arterial blood flow. This may have resulted from either an inflammatory process, from the pressure of an anteriorly prolapsed lens, or from a combination of these situations. A prolapsed lens could have pressed the irides between the anterior lenticular and posterior corneal surfaces. This might have expressed any blood and prevented any influx.

The following discussion concerns the gross appearance of lenses dissected from eyes excised following irradiation and used for biochemical or electrophoretic investigations. The lenses were faintly cloudy; the occasional whitened lenses observed at this time were not used for either electrophoretic or biochemical investigations. The region of microscopic bubbles observed beneath the anterior capsule of lenses dissected from eyes excised immediately following irradiation appeared similar to that observed in control lenses which had been immersed in normal saline for 30 minutes or more. The resultant appearance of slight cloudiness in either case may have been a non-specific response of the lens to injury, i.e., lenses from irradiated eyes may have exhibited thermal injury; lenses immersed in normal saline may have exhibited an injury which may have resulted from impaired metabolism (lack of glucose, etc.).

Certain of the eyes were enucleated and fixed following irradiation. The superior portion of the lenses from these eyes bore the imprint of the posterior surface of the iridial processes. This unwhitened, imprinted area may have been associated with an alteration of the subjacent lens cortex, and a concomitantly increased pressure of the iris on the lens surface. The situation of the altered cortex, demonstrable immediately post irradiation, appears analogous to the altered but unwhitened indented regions of lenses observed following 7 watt, 1.5 second focused irradiation

and subsequent fixation described in Chapter III. The apparent pressure of the iris on the lens may have been associated with corneal flattening. The torn lens capsule observed occasionally in these fixed lenses dissected from eyes enucleated immediately post irradiation may have resulted from a local shrinkage of the capsule collagen. The torn area was not apparent in unfixed lenses observed shortly following irradiation; either the tear was not noticed because of the transparent nature of the capsule and the underlying cortex or the involved region of the capsule was only structurally altered and tore during the fixation reaction.

The whitening of the suspensory ligaments of the lens (zonules of Zinn), noted in the superior portion of certain eyes shortly after corneal irradiation probably resulted from heat. The temperature of the zonules probably was increased by the vertical flow of heated aqueous humor. The inability of these thin avascular structures to conduct surface heat away from themselves either by blood flow or by conduction to deeper regions may have resulted in higher temperatures than that suffered by adjacent tissues such as the iris. Apparently, there was ensuing denaturation and coagulation. The relatively inelastic zonules (Dische, 1970) are normally inserted into the lens capsule at the equatorial region. The transient local heat shrinkage of the adjacent capsular collagen could have increased the tension of a series of these denatured zonules and caused the broken fibers frequently observed.

The liquefied cortical material observed without fixation two or three days post irradiation in the superior portion of the lenses may still have contained relatively normal proteins. This is inferred from the

observation that the liquefied region and the adjacent solid material of the lenses was indistinguishable in resiliency following fixation in glutaraldehyde. Assumedly, if the liquefaction were caused by marked proteolysis, the resultant fluid (amino acids and polypeptides) would not have reacted to the fixative in a manner identical to that of the parent proteins.

Normally, it takes deliberate effort to separate the capsule from the underlying cortex. Four to five days following irradiation spontaneous decapsulation occurred during dissection of the lens. This indicated a lessening of the attachment between the cortex and capsule. This may have been due to a tear or an altered area of the capsule, which might have permitted abnormal passage of aqueous humor into the subcapsular region. It appears, however, that entry of aqueous humor may result in lens whitening. This did not occur except in the superior region of the lens; however, the lens-capsular attachment apparently was lessened in all regions. Consequently, an alternate explanation is that the metabolism of the lens may have been altered in such a way as to weaken the capsular-cortical attachments.

Immunological factors may have been of importance in the lenticular capsule weakening or tearing. Heat-induced alteration of the lens capsule might have changed its immunological response. Kuck (1970a) reported that the lens capsule has a high concentration of polysaccharides and thus has been suspected as a source of antigens.

The cause of the delayed whitening of the superior region of the posterior lenticular surface observed four to five days post irradiation

is not readily apparent. One may assume that it resulted from thermal damage to the lens fibers. Mature lens fibers extend from the anterior sutural region across the equator to the posterior sutural region. It appears that the heating caused by the irradiation of the cornea raised the temperature of the anterior region of the lens to a higher level than that of the posterior region; therefore, one might expect the delayed whitening to be visible initially in the anterior region. The reasons for this apparently anomalous site of whitening in the posterior part of the lens is not understood.

The marked degree of total whitening and decreased volumes of the lenses observed six days post irradiation may have resulted from a number of factors. Although the exact mechanism is not clear, it probably involved interactions between aqueous humor and lenticular contents, the non-specific response of dead and dying cells in avascular tissue, inability to maintain metabolism of the fibers and proteolysis.

As discussed, the majority of the lenses used appeared relatively clear on *in vivo* inspection immediately post irradiation. Approximately ten percent of the eyes, however, contained obviously whitened lenses; the eyes from this latter group were not used for electrophoretic or biochemical investigations. This immediate whitening of the lens possibly resulted from excessive heating and may have been due to the following: 1) A reduced heat-dissipation of these eyes; 2) a comparatively higher pre-irradiation ocular temperature; and 3) increased irradiation of this group. Comparatively shallower anterior chambers and smaller iridial blood flow of slightly smaller eyes may have a lessened capacity for heat

dissipation. This may have resulted in a greater temperature elevation in these eyes during the irradiation. Alternately, although less likely, the whitened lenses may have been heated to a higher temperature not because of a greater temperature elevation per se on irradiation, but because of a higher pre-irradiation temperature. In this regard, Goldmann (1933a) stated that the amount of injury is dependent on the final temperature rather than the temperature increase per se. He reported that the ocular temperatures rise and fall with the temperature of the body. An ancillary investigation in this chapter showed that the body temperature decreased following the administration of the anesthetic agents. This decrease averaged approximately 1.5° C for the initial 30 minutes. Thus, if the interval between the administration of the anesthetic agents and the initiation of the irradiation were short, the pre-irradiation ocular temperature would have been higher than that in eyes of animals anesthetized for longer periods of time. This would have resulted in a final ocular temperature which was relatively higher than in the latter group. Although this latter mechanism must be considered, it probably is of minimal importance.

Electrophoretic Investigations

Operation of the electrophoresis unit in a refrigerator at 5° C may have prevented some protein denaturation by facilitating removal of heat evolved during the separatory process.

Maintenance of the lens homogenate temperature above 10° C during separation is said to prevent cold precipitation of γ crystallins (Kuck, 1970a). Although γ crystallin is the smallest of the crystallins

(M.W., 1.7×10^4), it is the least soluble. Kuck states that this low solubility does not appear to result from any unusual amino acid composition. Rather, he suggests that the folding of the molecule may expose a relatively large number of hydrophobic groups. This low solubility is apparently decreased to a critical point when the ambient temperature is brought below 10°C. Although the σ crystallins in the sample might have precipitated on application to the strips (5°C), they would have been resolubilized following the heat on electrophoresis. This reversibility of σ crystallin insolubility is attested to by the lack of material at the site of application following electrophoresis. Furthermore, young animals can develop "cold cataract" when their bodies are cooled sufficiently. On rewarming, the cataract disappears and the lenses are again transparent. This phenomenon is attributed to the relatively large percent of σ crystallin in young lenses.

Identical patterns were produced on simultaneous electrophoretic separation of soluble proteins from pairs of eyes of unirradiated animals. This indicated that the separatory procedures used were reproducible. The electrophoretic separation of soluble lens proteins from unirradiated rabbit eyes appeared identical to those shown by Mehta and Maisel (1966).

There were no differences in mobilities observed and in the patterns produced on simultaneous electrophoretic separation of soluble lens proteins of irradiated and control eyes sampled one day post irradiation. This suggested that there had not been any immediate gross alterations of the soluble proteins on heating due to irradiation, at least, alterations which grossly altered the electrophoretic pattern. Examination of the

electrophoretic pattern of soluble proteins from lenses heated to 60-70° C for 30 minutes showed a marked decrease of the mobility of all crystallins (Mehta and Maisel, 1966). If coagulated insoluble proteins were produced in the lens on CO_2 irradiation, however, it would either have been removed from the supernatant on centrifugation or would not have moved from the site of application on the electrophoretic strip.

The electrophoretic studies did not shed much light on the phenomenon of immediate lens indentation on heating. It appears that the reaction to electrophoresis of soluble proteins from a lens sampled immediately post irradiation was not grossly different from that of the control lens material. Furthermore, no difference was noted between electrophoresis of the semi-fluid material found beneath the lens capsule and that of normal cortical material. A difference might have been expected.

Comparison of this finding with the observation of indentations following fixation with aldehyde fixatives suggests that the heating may have exposed side chains (e.g., amino groups); glutaraldehyde or formaldehyde can react with amino side groups and form a cross linkage (Pearse, 1960). An increase in the number of these groups (NH_2-R) might not have altered the electrophoretic mobility of a protein in the alkaline pH buffer used, pH 8-9, because the amino groups would not be ionized at this pH.

Experiments carried out in an acid buffer might show differences in the soluble protein from control and irradiated eyes. These were not done.

The heat-induced indentation observed in unfixed decapsulated lenses may have been physical and not chemical in nature. It may have been the

result of extrusion of crystallins from the anterior terminal section of lens fibers, or a local removal of water normally present. If a quantity of soluble protein was extruded posteriorly within each fiber in the anterior lens region on localized heating, the ratio of lens fiber crystallin to lens fiber membranes in this "indented" region would have been altered. This possible alteration of the ratio of fiber membranes to contents in the heated area might have been a cause of the tinctorial differences observed between the indented area and the remainder of the fixed lens (as reported in Chapter III) following immersion in acid orcein. Orcein is said (Pearse, 1960) to possess positively charged groups which are necessary for its staining action. The orcein molecule is repulsed by tissue possessing a preponderance of positively-charged side groups. These groups may have been exposed (and thus been more reactive) at the low pH of acid orcein.

Differences were noted between the electrophoretic patterns of soluble lens material obtained from control and irradiated eyes when they were sampled two to three days post irradiation. Mobility of that assumed to be β and γ crystallins were somewhat lessened, whereas, that of α crystallin was not grossly altered. The β and α lenticular protein groups normally move slower than α crystallin. This suggests that either there were less negatively charged groups present in the β and σ proteins or that their molecular weights had increased. According to Davson (1969b) and Spector (1971), the β and σ crystallins normally contain a large amount of sulfhydryl groups. Kinoshita (1964) states that one of the purposes of lens glutathione is to protect labile protein sulfhydryl groups

from oxidation to disulfide bridges. A decreased concentration of GSH could therefore permit the production of inter- and intra-molecular disulfide bridges. The production of these bridges would either reduce the number of ionized hydrophilic groups on the protein surface or increase the molecular weight of the molecules or both. Consequently, there may have been a relationship between the lessened mobilities of these protein groups and the lower concentration of glutathione in the lens at that time.

Previous workers have electrophoretically separated normal rabbit aqueous humor without performing paracentesis; however, they were forced to pool lots of the liquid and then concentrate it prior to electrophoretic separation. Moore (1959) tabulated the comparative electrophoretic mobilities of both pooled aqueous humor and serum from the rabbit. They were similar except that no globulin was reportedly present in the aqueous humor (neither had fibrinogen). The mobilities of each band compared well (i.e., aqueous humor versus serum). Also investigations discussed by Moore show that the albumin concentration increased in aqueous humor following treatment with vasodilators. This situation may have occurred in the eyes in this investigation following paracentesis. Furthermore, all components of plasma proteins are present in the aqueous humor during acute ocular inflammation. Presumably this occurred in the irradiated eyes.

Comparison of the patterns produced by the soluble lens proteins from these irradiated and control eyes showed that although the fastest bands of the soluble lens protein had traveled similar distances, the slower protein bands from lenses of the irradiated eye had traveled noticeably shorter distances. The pattern produced by the plasmoid aqueous

humor appeared grossly similar to that produced by the soluble proteins from the control eye as well as having the characteristic bands of blood serum protein. In contrast, the pattern produced by the liquid from the anterior chamber of the irradiated eye did not resemble either that of plasmoid aqueous humor or the soluble lenticular material from either eye. The pattern appeared to be a composite of the patterns of lenticular proteins and the plasma proteins assumedly present in the aqueous humor as a result of ocular inflammation or trauma. This suggests that soluble proteins were leaking either into the aqueous humor through the capsule of the lens because of altered capsular permeability, or through a fissure in the capsule. Dische (1970) reported that the lens capsule contains glutathione. Possibly, the concentration of GSH in the capsule diminished along with that of the remainder of the lens. If so, diminution of the GSH might have increased the permeability of the lens capsule to crystallins. Furthermore, Epstein and Kinoshita (1970) have shown that a diminished concentration of GSH increases the permeability of the lenticular membranes.

Ascorbic Acid and Reduced Glutathione Determinations

Ascorbic acid in aqueous humor

Sampling of aqueous humor with the Hamilton microliter syringe from irradiated eyes was abandoned because of difficulties in proper positioning of the needle in the anterior chamber with a somewhat opaque cornea. Also, fibrinogen in the aqueous humor of such eyes clogged the syringe needle. The alternate method in which aqueous humor was expressed through the slit corneas of excised eyes permitted acquisition of adequate material for AsA determinations. The consistently lower AsA concentrations as well

as the relatively large differences between values measured in pairs of control eyes (*maximum difference*, 24%) indicated that this second method of sampling was not optimal. There did not appear to be an alternate method readily available, however. Any of the following factors could have decreased the AsA concentration in the aqueous humor in the control eye obtained by expression: oxidation of AsA, hydrolysis of the lactone ring of AsA, as well as uptake of the substance by ocular tissues.

Enucleation halted the blood flow and thus the influx of aqueous humor. There seemed to be an associated decrement of aqueous humor efflux, as the enucleated eyes did not appear flaccid at the time of sampling. Therefore, the volume of aqueous humor must not have been decreased appreciably. The quantity of AsA in the aqueous humor probably was normal at the time of killing and enucleation of unirradiated eyes. Following enucleation there may have been a certain percentage of the total aqueous humor AsA which was oxidized. (Adler, 1966, noted that data from assays of aqueous humor from dead eyes might be viewed with suspicion because of postmortem changes.) This was probably minimal as the ocular temperature was rapidly reduced with ice following excision.

There may have been a percentage of the AsA hydrolyzed at the pH 7.6 of normal rabbit aqueous humor (Davson, 1969). Hydrolysis of the lactone ring can occur in an alkaline medium with the resultant formation of diketogulonic acid (West, Todd, Mansion and van Bruggen, 1966). Furthermore, there might have been an uptake of AsA by ocular tissue in the interval between excision and sampling; also passage through and contact with the corneal stroma may have oxidized, hydrolyzed or removed some AsA from

the aqueous humor. It would be interesting to compare the concentrations of aqueous humor AsA sampled from dead eyes by this (expression) method with that sampled with a syringe.

The AsA concentration in the aqueous humor was decreased in all irradiated eyes sampled in this phase of the experiments. Unfortunately, only a small number of irradiated eyes could be sampled (see Table IV-1). Nevertheless, the relative concentration of AsA in the aqueous humor was decreased in eyes sampled at two hours post irradiation and there was a continual decrement in aqueous humor AsA sampled during the ensuing post irradiation period.

The decrement of aqueous humor AsA concentration in irradiated eyes (compared with that in control eyes) may have been due to several factors. Both the active transport from ciliary processes and the diffusion from iris stroma into the aqueous humor may have been decreased. The rate of AsA oxidation may have increased.

The small concentration of plasma AsA (1 mg%, Davson, 1969a) is said to be actively concentrated in the ciliary epithelium . AsA is then considered to be actively transported from the epithelium of the ciliary processes into the aqueous humor at the posterior chamber, resulting in a concentration of 20-30 mg% in the posterior chamber (ibid.); a lesser amount diffuses from the iridial vasculature. The concentration of ascorbic acid in the aqueous humor resulting from simple diffusion would probably equal that of the blood plasma (i.e., 1 mg% or approximately 1/20th of that of normal aqueous humor). Any decrement of the blood flow to this region would be reflected by a decrement of ascorbic acid available for

transport; hence, a decreased transfer of ascorbic acid into the aqueous humor. Initially the blood flow was halted only in the upper third of the iris and ciliary processes of irradiated eyes. This effect of excessive heat and pressure on the upper region of the anterior segment of the eye has been discussed. Secretion of ascorbic acid from this region is dependent on blood flow (Davson, 1969) and therefore it must have been abolished locally. Furthermore, if the temperature elevation in the anterior segment of the eye had been sufficient to abolish blood flow to the superior region of the iris, it is entirely possible that it might have also decreased the activity of the transport mechanism in the grossly unaffected inferior regions of the iris and ciliary processes. Therefore, immediately following irradiation, the secretion of ascorbic acid in the upper portion of the ciliary process may have been abolished because of a cessation of blood flow and at the same time the output in the remaining ciliary process was markedly lessened because of a thermally impaired secretory mechanism.

The total blanching observed in eyes approximately five days post irradiation has been discussed; this probably abolished any residual secretory activity.

The oxidation rate of the ascorbic acid in the aqueous humor may have increased following corneal irradiation. There are two causes which may have produced increased oxidation: 1) Following the sloughing of the corneal stroma only Descemet's membrane and the residual endothelial cells separated much of the anterior chamber from the ambient air; this could have permitted entrance of additional oxygen into the aqueous humor

thereby contributing to the oxidation of ascorbic acid and thus lessening its concentration in the anterior chamber. 2) Apparently ascorbic acid accumulates in injured tissue shortly after the insult and prior to the production of collagen. This may have reduced the concentration of aqueous humor AsA. Gould (1963) discussed the increased uptake of AsA by injured tissue.

Lens ascorbic acid following CO_2 laser corneal irradiation

The slope of AsA decrements with time in aqueous humor and in lenses of irradiated eyes appeared grossly similar. The ascorbic acid concentration of the aqueous humor, however, was decreased immediately following irradiation, whereas the lenticular concentration was initially normal and began to decrease after one day. A similar lag period in the decrement of AsA from the lens in comparison to that in the aqueous humor was reported by Hughes, Hurley and Jones (1971) in investigations on the scorbutic guinea pig.

Lenticular ascorbic acid

Lenticular AsA is obtained from the aqueous humor (Davson, 1969), (Kuck, 1970). In human and other eyes, there is a greater concentration of AsA in the lens than in the aqueous humor. This situation led van Heynigen (1969) to state that, "accumulation of ascorbic acid in the lens of some species suggests the participation of an active process." In the rabbit eye the concentration is greater in the aqueous humor than in the lens; in this species the lens could therefore obtain its AsA by simple diffusion. It is possible that the CO_2 laser irradiation of the cornea in

this experiment altered the permeability of the lens in such a manner as to depress the normal rate of AsA diffusion into the lens (Fig. IV-10, Table IV-1).

Kinoshita, Merola, Dikmak and Carpenter (1966) reported a lenticular AsA decrement following cataractogenic microwave irradiation. Since their aqueous humor AsA concentrations remained normal, one might conclude that the decrement resulted from a grossly impaired lenticular AsA transport system. These results could be explained on the basis of interference with a normal passive diffusion process. In several of our irradiated eyes, the lens AsA was higher, at that time, than the aqueous AsA. Considerably more must be known about the metabolism and metabolic rate of AsA in rabbit lenses in order to decide as to whether our data (lens AsA greater than aqueous AsA) support the possibility of an active mechanism for AsA transport into the rabbit lens, either alone or in conjunction with a passive process. Certainly, in most of our normal and control eyes, the aqueous:lens AsA ratio (taking into account our slit technique) would not be evidence for an active process. However, based on the above, the possibility of a mechanism for active lens AsA transport deserves further consideration.

Kinoshita, et al (ibid) reported that the decrement of lenticular AsA occurred 18 hours following the irradiation; the cataract did not appear until a week later. The aqueous humor AsA concentrations remained normal during this period, suggesting that the ciliary process epithelial secretory mechanisms had not been grossly impaired by microwave irradiation at this power density. Furthermore, they reported that lenticular concentrations of dehydroascorbic acid and diketogulonic acid did not increase concomitantly. They inferred from these findings that

the decreased AsA concentrations had not resulted from increased lens oxidation of AsA, at least to these aforementioned products. The relationship between a decrement of AsA and the production of cataracts requires further study.

The decrement of AsA observed in the lens in our investigations might not be due only to decreased transport, active or passive, into the lens, but to increased utilization of the AsA. According to van Heynigen (1969), hydrogen peroxide produced in the anterior chamber on oxidation of AsA by a light-catalyzed reaction can readily diffuse into the lens. Normally the hydrogen peroxide is reduced by lenticular reduced glutathione in the presence of glutathione peroxidase (the activity of catalase in the lens is very low or absent). With a decrement of lenticular GSH, the rate of peroxide reduction would be lessened. In the presence of AsA the hydrogen peroxide could react with and cleave unsaturated lipids (Hochstein and Ernster, 1964) which are normally present in the lens (Kuck, 1970). (Presumably, some of these unsaturated lipids make up the lens fiber membranes.) This cleavage would result in liquefying the lens constituents and a concomitant decrement in AsA. Since some lens liquefaction has been observed in these experiments, this might indicate that the above process of increased AsA utilization should be further considered. The liquefaction produced in the present investigation also requires further investigation. Whether these processes bear any relationship to the liquefaction observed in the early stages of hypermature or Morgagnian cataracts described by Cogan (1962) is speculative.

Membrane liquefaction may be related to the anomalous delayed whitening observed at the posterior of lenses following CO_2 laser irradiation of the cornea. Kuwabara (1966) stated that microtubules are common in the lens

fibers of the superficial cortex at the bow region of the lens. Although his microphotographs were made from tissue from human lenses, it is possible that similar structures exist in the rabbit lens and that these structures, like other subcellular organelles, contain lipids. The distance between the posterior portion of the elongating lens fiber and its nuclear region, as well as the distance of this portion from the epithelial cells increases with time. It is possible that certain necessary substances resulting from epithelial transport or from synthesis in the nuclear region may reach the posterior of the fiber through microtubules. If these pathways be lipoprotein in nature, they might be disrupted by excessive lipid peroxidation. Such a disruption might lessen the flow of metabolically necessary substances to the posterior of the affected lens fibers. Disturbed metabolism of the lens may result in decreased transparency (Duke-Elder, 1970). The resultant situation might be analogous to axon degeneration, distal to the separation site of a severed nerve.

Lenticular reduced glutathione concentration

Inspection of the graph of GSH concentration verus time following irradiation (Figure IV-11) shows a monotonically decreasing decay curve. One to two days post irradiation the concentration had not decreased greatly; in the ensuing days, however, the rate of the concentration decrement increased markedly, and by the fifth to sixth day post irradiation the concentration was nearly zero.

The greater part of this decrement may have resulted from one of three mechanisms: 1) decreased GSH synthesis; 2) diffuse of GSH into the

aqueous humor; or 3) markedly increased oxidation of glutathione (GSH to GSSG).

With regard to decreased GSH synthesis, the following is a consideration of possible factors which might have resulted in a decrement. Aqueous humor in the anterior chamber heated the iris during CO_2 laser irradiation of the cornea. It may then have flowed through the pupillary aperture into the posterior chamber and heated the structures in this area, e.g., the ciliary processes and the lens. Furthermore, heat may have been conducted directly into the posterior chamber. The heated fluid apparently ascended and heated the superior portion of the iris, ciliary processes and lens to a greater level than the remainder of these tissues, causing injury to these regions. Thus, the activity of certain processes or mechanisms necessary for normal aqueous humor formation required for normal lenticular metabolism were apparently diminished during CO_2 laser irradiation of the cornea.

According to Kuck (1970b), the output of the normal ciliary processes provides the aqueous humor with substances apparently required by the lens for metabolism, such as amino acids, glucose and AsA. The effects of the lack of blood flow to a portion of these ciliary processes and the possible impairment of transport mechanism in the remaining ciliary processes has been discussed in this chapter. Amino acid and glucose secretion into the aqueous humor (including those amino acids necessary for GSH synthesis in the lens) may have been similarly depressed following irradiation; however, they were not assayed in this study.

Even if the aqueous humor concentrations of the amino acids had been adequate, the mechanism for entrance of these substances into the injured

lens and for GSH synthesis may have been depressed by thermal injury. Also, there may have been thermal inactivation of both the lenticular enzymes necessary for GSH synthesis as well as those involved in the production of the ATP required for the synthesis process. Thermal inactivation of involved enzymes may have been most marked in the superior region of the lens. If the involved enzymes of the remainder of the outermost cortical regions of the lens had not been inactivated during corneal irradiation, at least their activity might have been lessened. This could have greatly reduced or abolished lenticular GSH synthesis during CO_2 laser irradiation of the cornea.

GSH itself, might be responsible for maintaining the integrity of enzyme systems necessary for GSH synthesis, or for transport of the amino acids into the lens. The decreased concentration of lens GSH in time may have contributed to the demise of any residual transport mechanisms which might have sustained lenticular glutathione in the reduced state. For example, Kinoshita (1964) said that the relatively high lens concentration of GSH might serve to maintain certain susceptible sulfhydryl groups in a necessary state of reduction. Sulfhydryl groups are said to be necessary for the activity of certain dehydrogenases and ATPases, which may be significant in some aspect of GSH synthesis.

If GSH synthesis in the lens were a simple first order reaction and had been completely abolished on irradiation, and GSH degradation rate were dependent on GSH concentration, one might expect an exponential decrease of GSH concentration with time. If the rate constant for conversion of GSH to GSSG had remained equal to that in the normal lens, and

this conversion were the major factor in the removal of GSH, then the rate constant would have been equal to that for GSH synthesis. Rate constants have been determined for GSH synthesis in the rabbit lens, but only for a specific concentration of lenticular glutathione. Kinsey and Merriam (1950) reported that one-half of radio-labeled glycine in GSH was replaced every 29 hours (rate constant of 2.4%/hour). (The half life may be determined from the rate constant by $C = Co\ e^{-kt}$, $C = 50\%$, $Co = 100\%$, k = the rate constant and t = time.) Reddy, Klethi, and Kinsey (1966) investigated the rates of incorporation of radio-labeled glycine and of glutamic acid into GSH in lenses of young rabbits. They reported an average turnover rate of 1.8%/hour for both glycine and glutamic acid (half life of approximately 38 hours). Although in our investigations the GSH concentration decreased post irradiation, the decay curve did not appear to be exponential; indeed, it appeared to decrease most rapidly during the one to three day period. If the normal rate of conversion (GSH to GSSG) increased as the GSH concentration decreased during these first three days, a stoppage of synthesis might result in a curve in the first three days similar to that observed. However, our data is limited; and we do not think the conversion of GSH to GSSG increased with decrease in GSH concentration.

With regard to increased diffusion of GSH, if we assume that the decrement of lenticular GSH resulted from an increased permeability of the lens to reduced GSH, we must assume that the irradiation markedly altered the lens permeability to the tripeptide. Normally, (Cole, 1970) there is no reduced glutathione present in the aqueous humor. In the present experiment, initial measurements were made in the aqueous humor

for GSH. None was found; possibly if the assays were carried out with a more sensitive method (e.g., the glyoxalase method), this substance might be found in the aqueous humor. Nevertheless, if the permeability of the lens to GSH were increased it would cause a decrease in lenticular GSH, and also, it should alter the ratio of lenticular GSH to GSSG.

Oxidized glutathione (GSSG) can readily pass from the lens into the aqueous humor. Srivastava and Beutler (1968) have suggested that the value of the steady state concentration of GSH measured in the normal lens probably exists in part because of this selective permeability to oxidize glutathione.

Assume that the GSH synthetic rate was unchanged following irradiation, but the rate of oxidative conversion of GSH to GSSG increased. If this were so, the loss of GSSG from the lens would have markedly increased if one assumes that the lens membranes were unchanged in their permeability to GSSG. Such conversion could have occurred because of increase oxygen diffusion into the lens. This might have resulted from an increased oxygen concentration in the aqueous humor, resulting from a thinning of the irradiated cornea several days post irradiation. Also, altered permeability of the lens capsule following irradiation might have permitted an increased passage of oxygen from the aqueous humor into the lens. Furthermore, a large tear in the capsule would have had a similar effect; the degree of oxidation probably would have been maximal in the immediately vicinity of the tear. Increased oxidation of GSH would have resulted in an altered ratio of GSH:GSSG in the lens from a normal value of approximately 10:1 (Waley, 1969).

The decrease in GSH concentration may have been due in part to increased oxidation. Although the slope of the curve for GSH concentration as a function of time in the present investigation cannot be accurately determined, it appears that there was a greater rate of decrement of GSH in the period after one day post irradiation. During this period, there was a marked amount of contact between the aqueous humor and the cortical material, as previously discussed. In a study cited by Prince and Eglitis (1964b), it was reported that the oxygen uptake of a lens markedly increased following capsular laceration and subsequent contact of the lens cortical material and the surrounding aqueous humor. The "equivalent" of a capsular laceration may have occurred several days following irradiation. This would have resulted in a contact between lens material and the surrounding aqueous humor and resulted in an increased GSH decrement rate.

Five days following CO_2 laser irradiation of the corneas, the lens GSH concentration was markedly reduced; however, the GSSG concentration was also greatly lessened. The sum of GSH + GSSG in the lens from the irradiated eye was much less than that of the contralateral control. There was no evidence of increased glutathione oxidation, as the ratio of lenticular GSSG/GSH in the irradiated eye approximately equaled that of the contralateral control eye (both were approximately 5%).

Inspection of the data of the needled lenses suggested relatively unimpaired GSH synthesis, but an increased rate of GSSG production. As there is no reported synthetic pathway for the direct production of GSSG from the component amino acids, the increased amount of GSSG in the needled

lenses must have been produced by the oxidation of GSH. Although the concentration of GSH + GSSG in the needled lenses approximately equaled that of the contralateral control lenses, the GSH:GSSG ratio had decreased and was quite different from that in the control lenses (e.g., the percentage of total glutathione (GSH + GSSG) present as GSSG in the needled lenses had increased by 50 to 100% over that in the control lenses).

Consequently, in the irradiation investigations there did not appear to be an increased conversion of GSH to GSSG, whereas, in the needling experiments there was an increased conversion. Therefore, it appears that the decrement in GSH observed in the biochemical determinations made in the major portion of the investigation (Figure IV-11) was not mainly due to increased oxidation. Furthermore, it does not appear that it was due to diffusion of GSH into the aqueous humor. Therefore, it appears that the decrement in GSH concentration observed resulted mainly from a decreased synthetic rate.

It is possible that all three mechanisms were active to an extent—decreased synthesis, increased diffusion and increased oxidation. Radioactive tracer studies may assist in determining the extent to which GSH decrement observed in the injured lenses was due to decreased synthesis of GSH.

It should be noted that the GSH studies were done on 1 1/2 to 2 lb rabbits whereas the GSH/GSSG determinations were carried out on 5 lb animals in order to obtain sufficient lens material. Despite the differences in weight of these test groups, it is probable that the conclusions based on the larger animals with regard to the mechanism for decrease in GSH concentration are valid for the studies on the 1 1/2 to 2 lb rabbits.

SUMMARY AND CONCLUSIONS

The purpose of this thesis has been the investigation of the effects of carbon dioxide laser irradiation of the cornea on the anterior segment of the rabbit eye. An attempt has been made to correlate temperature elevations caused by irradiation with previously reported tissue changes—both those resulting from direct irradiation, as well as internal alterations apparently resulting from intraocular temperature and pressure elevation caused by heat flowing from the irradiation site. Awareness of some of these findings should provide insight into the nature of injury occurring in an accidental exposure of the human eye to CO_2 laser irradiation. Furthermore, the findings suggest that the CO_2 laser can be used as a tool for the investigation of the responses of ocular tissue to controlled surface heating.

During the primary phase of these investigations steady-state surface temperature elevations were measured during continuous carbon dioxide laser irradiations of the entire corneal surfaces of adult rabbits.

These measurements showed that the maximum corneal temperature elevation on continuous 100 mW/cm^2 irradiation averaged 5.5°C. The average maximum corneal surface temperature elevation measured during irradiation at a level sufficient to produce permanent injury (200 mW/cm^2) was less than 10°C. Consequently, temperature elevations of less than 10°C may cause permanent injury to corneal and other ocular tissues. The average maximum corneal surface temperature elevation at 380 mW/cm^2 was less than 15°C. Fine, Berkow and Fine (1969) reported permanent corneal alterations which clinically resembled human band keratopathy on irradiation

at this level. This finding of alterations resembling band keratopathy following irradiation which produced temperature elevations of about 15°C suggests that the CO_2 laser may be used to investigate some factors affecting the development of band keratopathy. Furthermore, it suggests that heating the rabbit cornea to a temperature of about 50°C may result in an alteration resembling band keratopathy.

At power density irradiation levels between 20 and 300 mW/cm^2, there appeared to be a linear relationship between the steady state aqueous and corneal surface temperature. Between 300 and 600 mW/cm^2, there was a decrease in the rate of rise of steady state aqueous temperature relative to the corneal surface temperature. This occurred at aqueous temperature ranges between 40 and 47 C. For aqueous temperatures beyond 47°C, the slope of aqueous to corneal temperatures was higher than in the above intermediate zone. The data suggest that there is an active cooling mechanism probably related to the iridial vasculature responsible for attempting to maintain constancy of aqueous temperature, when the aqueous temperature rises above 40°C. Further basic study of this cooling mechanism and its relationships to similar mechanisms in other regions is warranted. Also, the effects of drugs on this cooling mechanism and, therefore, its probable effect on iridial blood flow and possibly blood flow in other regions could be evaluated using the methods described in this thesis.

In addition, the data obtained regarding the relationship between the steady state aqueous and corneal surface temperatures should permit such aqueous temperatures to be estimated by measuring the corneal surface temperature, in further studies on continuous CO_2 irradiation

of the eyes of rabbits. This data may permit estimates to be made regarding some thermal properties of the cornea, in vivo. Because of the similarity in size between human and adult rabbit eyes, the data, in conjunction with corneal surface temperature measurements, might provide some estimates of temperatures in the aqueous of human eyes exposed to elevated ambient temperatures. Furthermore, the data suggest that corneal surface temperatures (in conjunction with tonometer measurements) might provide an indication of the relative effects of drugs on the iridal vasculature, in studies on man.

The anterior chamber of the eye may be considered as a filled compartment, with a possibly high resistance to inflow and outflow. Heating of the aqueous humor could result in a concomitant increase of anterior chamber pressure. Therefore, in the second phase of this investigation simultaneous anterior chamber temperatures and pressures were determined during corneal power density irradiations of threshold values (100 mW/cm^2) and greater. During 100 mW/cm^2 irradiation the intraocular pressure did not increase and the temperature of the anterior chamber increased 3°C to a new steady-state level. At irradiation levels of 350 mW/cm^2 the temperature rose during the initial six minutes of irradiation to a maximum of 12°C, and the pressure increased by 20 mm Hg after 18 minutes of irradiation. It is possible that the aqueous humor flow rate was increased at these elevated temperatures and pressures. The effect of increased aqueous temperature and intraocular presure for aqueous turnover requires further investigation.

Pulsed 6-7 watt irradiations of the center of the cornea caused transient aqueous humor temperature and pressure elevations. The

maximum temperature elevation occurred directly beneath the site of corneal irradiation; that is, along the axis of the beam irradiating the surface. The temperature and pressure elevations were a direct function of the pulse duration; however, protracted irradiations resulted in corneal perforations with a resultant expulsion of heated aqueous humor and an immediately precipitous decrease of pressure. Carbon dioxide laser pulses at 6-7 watts for 1.5 seconds flattened the cornea and caused concomitant anterior chamber temperature increases of approximately 25-35°C and pressure elevations of approximately 50 mm Hg. Following enucleation of such eyes and subsequent fixation in glutaraldehyde or formalin, an unwhitened indentation of the anterior surface was observed, as has been previously reported. Examination of eyes enculeated and fixed following shorter irradiation did not show such lens indentations. Dissections of eyes enucleated and fixed following protracted irradiation which perforated the cornea showed large whitened indentations on the anterior lens surface. Consequently, suprathreshold non-perforating laser irradiation may result in increased pressure as well as temperature in the anterior chamber.

Furthermore, above a certain level of transient pressure and temperature elevation in the anterior chamber, changes can be produced in the rabbit lens which may not be observed in vivo but are evident only following fixation in media such as glutaraldehyde or formalin as an unwhitened indentation.

The actual site of the injury responsible for the unwhitened indentation observed on fixation has not been determined. Our studies [illegible] that the site of injury may, in part, be in the lens fibers.

Our results also show that the temperature and pressure elevation observed in the anterior chamber is not necessarily due to heating of the aqueous itself, but is, in part, associated with corneal flattening, which apparently results when the corneal stroma is heated to a critical shrinkage temperature (60-70°C). During this transient flattening, injury to the iris may also occur.

Therefore, in the event of accidental suprathreshold CO_2 injury of the eye in man, attention must be directed to the lens and iris as well as to the cornea—the site of absorption of the irradiation.

Changes in the protein concentration of the aqueous humor were measured in conjunction with the above anterior chamber temperature and pressure investigations. On irradiation of the cornea anterior to the iris, the anterior chamber became a deep blue following previous intravenous injections of Evans blue. Only slight coloration of the aqueous was observed following focused irradiation of the cornea over the center of the well-dilated pupil. On spectroscopic determination of aqueous protein, the concentration was maximum following irradiation over the center of the iris. Consequently, suprathreshold CO_2 laser or thermal irradiation of the cornea over the iris region can cause injury to the iridial vasculature.

The third phase of these studies was carried out in an attempt to determine the site of lens injury discussed above and to measure some possible lens biochemical changes which might occur on suprathreshold CO_2 laser irradiation. Irradiation (1.5 watts, 8 mm diameter area, 90 seconds) of the cornea of the weanling albino rabbit caused a clouding and temporary flattening of the cornea, a permanent blanching of the

superior region of the irides and a faint clouding of the lens. In several hours the curvature of the cornea had returned to apparent normalcy; however, the transparency of the cornea had decreased. The mid-anterior surface of the cornea sloughed off during the following days; the greater thickness of the corneal stroma was lost in this process. Nevertheless, there was some fluid in the anterior chamber; apparently Descemet's membrane was intact. The superior region of the irides and attached ciliary processes remained blanched; four or more days post irradiation the remainder of the iridial tissues became blanched.

Several days post irradiation the lenses showed regions of subcapsular liquefaction and some whitening of the superior half of the posterior region. On dissection, the lens capsule spontaneously separated from the cortex. Six days post irradiation the remaining lens material was whitened.

Initially, following CO_2 laser irradiation of the corneas of these weanling rabbits, there was no observable difference in the electrophoretic pattern of the lenticular material from irradiated and contralateral control eyes. Three or four days following irradiation, however, the electrophoretic patterns of the lenticular proteins differed from that of the controls. The pattern produced by the crystallins from the irradiated eyes showed lessened mobilities when compared with that of the contralateral control eyes. The greatest decrement was observed in the β and γ crystallin groups. The mobility of the α crystallins was either grossly unchanged or only slightly decreased.

Although lens indentations can be demonstrated on fixation immediately following irradiation, there was no observable alteration in the electrophoretic pattern of lenticular material at that time. Consequently, although we do think that there may be immediate injury to the lens fibers, we were not able to show this by electrophoresis. A more sensitive method of determining subtle alterations of soluble proteins might be necessary to assess this immediately change of the lens in response to heating.

The weights of the lenses were essentially identical for the first five days following irradiation. It is possible that the protein content of the lenses may have decreased and this was counterbalanced by an increased water content. During the next day, the comparative weight of the lenses from irradiated eyes decreased markedly. The capsule spontaneously separated from the remainder of the lens at about 3 to 5 days. The decrease in lens weight occurred at a time coincident with or following the observation (by electrophoresis) of lenticular proteins in the aqueous humor. Possibly, more sensitive methods may have shown the presence of lens protein in the aqueous humor previous to four days. The presence of lens protein in the aqueous suggests that thermal injury may alter lens permeability and also result in release of lens protein, possibly associated with a capsular tear; an immune reaction may result.

During the first day post irradiation, the concentration of both ascorbic acid (AsA) and reduced glutathione (GSH) in the lenses of irradiated eyes did not appear to be altered. The concentrations decreased by three days following irradiation. The decrement of the AsA and the GSH appeared nearly identical; by six days post irradiation, the concentrations were low.

Ancillary investigations indicated that the decreased lenticular GSH concentration observed in irradiated eyes resulted from a decreased synthesis rather than from increased conversion of GSH to GSSG.

The AsA level in the aqueous humor appeared to decrease and remainded low in comparison with the contralateral control eye. This decrease of AsA in the aqueous humor may have been due to heat injury to the AsA transport mechanism in the ciliary processes and to decreased iridial blood flow evidenced by the blanched irides.

Normally, in the rabbit eye, the ascorbic acid concentration is higher than that in the lens. This suggests a passive transport (or diffusion) mechanism. In our studies, it was necessary to use a corneal slit technique rather than a direct sampling of the aqueous humor with a syringe. This resulted in lower AsA concentration values for the aqueous humor than obtained with the Hamilton microliter syringe _in vivo_. If this factor is taken into account, it appears that in most of our normal and control eyes, the aqueous humor AsA concentration was greater than that in the lens. This tends to support the accepted impression that the AsA transport mechanism into the rabbit lens is passive. However, in several of the irradiated eyes, the aqueous humor AsA concentration appeared to be considerably lower than that in the lens. Although considerably more must be known about the metabolism of AsA in rabbit lens in order to decide as to whether or not an active process exists for transport of AsA into the lens, either alone or in conjunction with a passive process, the data above may suggest that investigations are warranted into the possible presence of an active process for transport of AsA into the rabbit lens. Although unlikely,

one might consider whether it is possible for an active transport process to be initiated on thermal injury.

A relationship may have existed between the observed altered electrophoretic pattern of crystallins and the decrease in lenticular GSH concentration. Both the decreased mobilities of the crystallins and the decreased GSH concentration occurred after a similar delay following irradiation. The β and γ crystallins which exhibited the greatest decrement in mobility normally contain the greatest number of sulfhydryl (SH) groups. In contrast, the α crystallins which normally contain a comparatively small number of -SH groups, showed a correspondingly minimal alteration in electrophoretic mobility. Since GSH may be responsible for normally maintaining the β and γ crystallin -SH groups in the reduced state, thereby minimizing the rate of formation of disulfide bridges, the decrement of GSH in the lens may have been responsible, in part, for the alteration of the β and γ crystallins observed as changes in electrophoretic mobility. The significance of GSH in the lens for the maintenance of the crystallin -SH groups in the reduced state requires further investigation.

In addition, another role which had been attributed to GSH is maintenance of the reduced state of -SH groups in the active sites of some enzymes (ATPase and dehydrogenases). The decrease in GSH may, therefore, have affected the activity of these enzymes. Consequently, on thermal injury, a delayed cataract may result due to interference with the lens metabolism rather than due to direct denaturation and coagulation of the lens.

APPENDIX 1

THE CARBON DIOXIDE LASER

Figure A-1-1 is a simplified diagrammatic representation of the laser set-up and its associated gas supply, vacuum system, electrical circuitry, cooling system and electrically activated shutters. A diagram of the laser is shown in Figure A-1-1.

It consists of an 80 centimeter long double-walled, water-cooled glass tube with a 2 centimeter inside diameter fitted with ground glass connectors at either end. Mating end pieces were cut at the Brewster angle for sodium chloride at 10.6μ. These sodium flats were 2 inches in diameter and 1/4 inch thick and had optically flat parallel faces. The latter were connected to the Brewster angle faces of the connectors with Dow Corning clear silicone rubber cement. When the laser was not in use, the flats were kept clean and dry by enclosing them in plastic bags heated with 60 watt bulbs 6 inches distant. The high voltage electrodes were sealed into the end pieces perpendicular to the tube axis. Power was supplied to these electrodes from the power transformer (Jefferson Luminous Tube high power factor transformer; 120 volts a.c. input, 15,000 V output). The output power was adjusted by means of a Variac in the transformer primary circuit. The electrodes were air cooled during operation with two fans (Muffin Fans, 14W, 120V, Rotron Mfg. Co., Woodstock, New York).

Gas (5% CO_2, 15% N_2, 80% He) was supplied to the plasma tube through Tygon tubing. During operation of the laser, a pressure of 2.5 torr was maintained in the plasma tube by means of a vacuum pump (Kinney Model KD-

8 High Vacuum pump, Kinney Vacuum Division, New York Brake Co., Boston, Mass.). The vacuum pump was connected to the laser cavity by means of 1 1/2" o.d. Tygon tubing; a pressure gauge and a needle valve in the line permitted pressure adjustments.

The chamber was cooled during operation by cold running tap water which was circulated through the water jacket surrounding the plasma chamber; the inflow and efflux connections were made with Tygon tubing.

The laser cavity was formed by a 100% reflecting gold coated surface mirror with a 4.7 meter radius of curvature and a flat germanium reflective (65%) disc as the coupling reflector. The two reflectors were 1.5 meters apart and were mounted in adjustable mirror mounts (Lansing Model GD-253 Mirror mounts). As the laser tube was physically separate from the end windows, the sodium flats coule readily be replaced as needed. Also, shutters for the control of the pulse length were readily inserted into the cavity. The shutters were operated by rotary solenoids (Ledex) connected to a suitable energizing source.

The pulse length was adjusted by varying the capacitance of the circuit triggering the shutter solenoids. The pulse was calibrated as follows: the laser rear cavity reflector window was removed and the beam from a helium-neon laser was directed along the axis of the cavity. A photocell was situated so the beam impinged on its light-sensitive surface; the output of the photocell was monitored on an oscilloscope with a calibrated time sweep. With this arrangement the total capacitance in the circuit could be adjusted so that the triggering of the circuit would result in the pulse length desired.

The laser was readily aligned by use of the helium-neon laser fitted with a pin-hole aperture. The beam was directed through the center of the NaCl flat and along the axis of the plasma tube following removal of the rear mirror (gold coated front surface mirror). The HeNe beam impinged on the center of the germanium flat front end window which reflected the beam. The germanium mirror was then adjusted by means of the mirror mounts until the impinging beam was normal to the surface and was reflected onto the pinhole aperture. The rear mirror was then replaced without altering the HeNe laser's position, and adjusted by means of mirror mounts until the impinging beam was normal to the flat rear surface. This was sufficiently accurate to provide initial alignment.

The CO_2 laser beam was observed on Thermofax paper. The power detector (Coherent Radiation Model 201) was positioned so the output beam impinged on the center of its sensing element. The laser was tuned for maximum power output by varying the voltage of the transformer primary and adjusting the gas flow and pressure and fine tuning the end window.

APPENDIX 2

METHOD FOR THE ELECTROPHORETIC SEPARATION OF OCULAR PROTEINS
(Modified from Testa et al, 1968)

The rabbit lenses were removed as described previously and homogenized in 3.0 ml of Tris buffer 10^{-2}M pH 7.6 containing EDTA 10^{-3}M at room temperature. The homogenates were centrifuged at 28,000 × g (gravity) for 60 minutes at 10°C (Beckman L 2 ultracentrifuge) in the 50 head at 22,000 rpm. Ten lamda of the 28,000 × g supernatant or of the aqueous humor were applied 4 centimeters from the end of a labeled strip of cellulose polyacetate (Sephraphore III, 1 × 6 inch strips). These strips had previously been immersed for 24 hours in the electrophoresis buffer (Gelman high resolution buffer No. 51105-Tris Barbital pH 8.8, ionic strength 0.03). For sample application the cellulose polyacetate strips were aligned on sections of filter paper.

Samples from the same animal (lens and aqueous humor from control and irradiated eyes) were always run simultaneously; following sample application near one end, the strips were aligned in the Gelman electrophoresis chambers so that each end was equally immersed in the buffer solution and all strips were parallel. The sample ends were immersed in the cathodic buffer reservoir. The electrophoresis units were maintained and operated in a laboratory refrigerator at 5°C. After the strips were in place and the unit covered, 350 volts of potential were applied across the strips for 60 minutes (5 - 7 mA DC). At the end of the 60 minutes the voltage was removed, the strips were taken from the electrophoresis unit and floated, then submerged in a fixing stain for

10 minutes (Beckman fixative dye solution Ponceau S 0.5 gm TCA and 7.5 gm sulfosalicylic acid in 250 ml of distilled water). The strips were removed from the staining solution and the excess stain removed by serial passage through 5% acetic acid solution. The strips were aligned and the ends taped to a sheet of heavy filter paper; then the distance between the point of application (origin) and the bands measured and compared.

APPENDIX B

ASCORBIC ACID DETERMINATION
Visual Method of Roe (1954)

Sodium 2,6-dichlorophenolindophenol (DCP) is blue in alkaline media and pink in acid. Reduction of DCP results in a colorless leuco form. The DCP is reduced by the ascorbic acid in the solution—the ascorbic acid being oxidized. When the ascorbic acid is used up, the solution turns pink in the acid medium.

Reagents

The reagents for this assay procedure were prepared as follows. The DCP solution was made by dissolving 50 milligrams of Sodium 2,6-dichlorophenolindophenol with gentle warming in 150 ml of glass distilled water; 42 mg of sodium bicarbonate was added and dissolved. After cooling, the solution was decanted into a volumetric flask and the final volume adjusted to 200 ml. This solution was maintained in a light resistant sealed glass container at 5°C; the unused portion was discarded after one week. The ascorbic acid solution (AsA) was made by dissolving 50 mg of ascorbic acid (Fisher reagent) in 100 ml volumetric flask with 5% Trichloracetic Acid (TCA) (100 ml final volume). A 2.0 ml aliquot of this solution was diluted with sufficient 5% TCA to a final volume of 100 ml (standard AsA solution). The solutions were prepared prior to each determination and were maintained in light resistant capped glass containers in crushed ice.

Ascorbic Acid Standard Curve

The standard curve was made using the results of the following procedure. The volumes of the standard AsA 5% TCA solutions were adjusted

to final volumes of 3 ml by the addition of 1 ml of water and sufficient TCA, for example, 2.0 ml of AsA solution and 1.0 ml of water as a blank, or 0.5 ml of AsA solution, 1.5 ml of 5% TCA and one ml of water. The 3.0 ml containing the acid and TCA were titrated in a 10 ml glass beaker placed on a piece of white filter paper on a magnetic stirrer. The solution was constantly stirred during titration by the interaction of the magnetic stirrer and a 3mm long Teflon-coated "flea" in the beaker.

The solution in the beaker was titrated as follows: the DCP solution was delivered beneath the surface of the ascorbic acid solution through an "L" shaped glass tube whose other end was mated with the ground glass tip of a calibrated glass syringe. The syringe was immobilized and the plunger advanced by a micro-buret-gauge (Manufactured by the Microburet Instrument Co., P. O. Box 884, Cleveland, Ohio). Advancement of the piston moved the plunger of the calibrated syringe (SiY). The total distance was recorded on the gauge of the apparatus; each 1/1000 inch equalled one microliter of solution delivered from the syringe. A standard curve was made of total number of microliters of DCP solution versus micrograms of ascorbic acid.

Figure A-2 is a plot of a standard curve for ascorbic acid. The ordinate is in microliters of 0.025% 2,6-dichlorophenolindophenol (DCP) solution; the abscissa in micrograms of ascorbic acid (AsA). The plot is linear; however, it does not pass through the origin but intercepts the Y axis at 22 microliters of DCP solution. This quantity of DCP is transformed from blue to red by the acidity of the TCA used as a solvent for the AsA.

Ascorbic Acid Determinations

For ascorbic acid determinations either the total deproteinized supernatant from the aqueous humor, or 1.5 to 2.0 ml of the lens homogenate acid filtrate was used. The final volume of either was adjusted to 3.0 ml. The diluted aliquot was titrated as described in the assay procedure and the ascorbic acid content (Y) determined from the standard curve. The number of micrograms of AsA in the aqueous humor aliquot were divided by the uL of original material; this value uG/uL was multiplied by 100,000. This value was mg% of ascorbic acid.

The quantity of AsA in the lens in micrograms was

$$y \times \frac{3}{(\text{volume of filtrate assayed in ml})},$$

where the original homogenate was made by using 3 ml of TCA solution. The concentration in mg% was

$$y \times \frac{3}{(\text{volume of filtrate assayed}) \times (10) \times (\text{lens weight in grams})}.$$

Discussion

Roe (1954) described two methods for the determination of ascorbic acid by the use of 2,6-dichlorophenolindophenol (DCP). The reduction product of blue DCP is colorless. In the spectrophotometric method excess of DCP was admixed with the ascorbic acid solution. The lessened optical density of the remaining blue color was determined immediately following the DCP ascorbic acid reaction. By assaying a series of known amounts of ascorbic acid, a standard curve was prepared. The sensitivity

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of this method was adequate; however, this method would have necessitated the use of a second spectrophotometer in our case since we assayed the GSH by a spectrophotometric method. Also, the spectrophotometric technique for AsA required rapid manipulations between the initiation of reaction and the optical density determination. Therefore, this method was not employed in this study.

Determination of ascorbic acid by the visual titration method was simple; good reproducibility was obtained after one had performed repeated assays of known amount of the compound prior to each assay series. In this manner the eye of the investigator performing the titrations was attuned to the development of the same hue or tint as an end point.

DCP (2,6-dichlorophenolindophenol) is not a specific determinant for ascorbic acid as it can be reduced by other oxidizable groups (Roe 1954). For example, sulfhydryl groups (present in some amino acids and proteins) can be oxidized by DCP; however, in the presence of relatively large hydrogen ion concentrations (pH less than 4), the rate of oxidation is very slow and is slight if the titrations are done rapidly. The assay of ocular material in this study eliminated the main source of error, as the assays were performed on trichloroacetic acid (TCA) filtrates. These filtrates were essentially protein free and had large hydrogen ion concentrations (pH less than 3). Therefore, rapid titrations of samples performed in nearly protein free acid media held interference to a minimum.

APPENDIX 4

ASSAY PROCEDURE FOR REDUCED GLUTATHIONE
by the method of Grunert and Phillips (1951)

In this method, glutathione (GSH) and nitroprusside (NP) react forming a complex which is rose-colored in an alkaline medium; the presence of cyanide stabilizes the complex and retards fading of the color. The absorbance of the reaction product at 520 nm is a function of its GSH content. The method used was modified by utilizing a saturated solution of sodium chloride in place of solid sodium chloride as specified by Grunert and Phillips. Furthermore, proportionately smaller volumes of all reagents were used because of smaller (~ 6ml) cuvettes used.

The following reagents were prepared:

GSH Stock Solution

Glutathione (sigma grade B, lot 788-0604)	60mg
Distilled water to	100ml

GSH Working Solution

GSH stock solution plus 10% TCA 1:1

10% Trichloroacetic acid (TCA)

5% TCA solution (10% TCA in distilled water 1:1)

Saturated NaCl (at room temperature) (SS NaCl)

2% NP solution

Sodium nitroprusside crystals	1gm
Glass distilled water to	50ml

CN solution 0.357%

NaCN	375mg
$Na_2CO_3 \cdot 7H_2O$	18.6g
Glass distilled water to	100ml

The GSH stock solution was prepared immediately prior to assay and kept in a brown bottle covered with aluminum foil and placed in crushed ice.

The NP solution was also kept in darkened bottles and placed in the refrigerator at 5°C. The CN solution was kept in a polyethylene bottle at 5°C.

Preparation of a Standard Curve for GSH

GSH (working sol.)	5%TCA	H_2O	SS NaCl	NP	CN	Designation
0.0	0.4	1.6	3.0	0.5	0.5	Blank
0.1	0.3	1.6	3.0	0.5	0.5	30 μG GSH
0.2	0.2	1.6	3.0	0.5	0.5	60 μG GSH
0.3	0.1	1.6	3.0	0.5	0.5	90 μG GSH
0.4	0.0	1.6	3.0	0.5	0.5	120 μG GSH

The GSH and 5% TCA were allowed to stand at room temperature with 3.0 ml of SS NaCl for 10 minutes. Then 1.6 ml of water was added, followed by additions of 0.5 ml each of nitroprusside (NP) and cyanide (CN) solutions. The mixture was capped with Parafilm, mixed by inversion and the optical density (O.D.) immediately determined and recorded. The standard curve was made by plotting the O.D. at 520 nm versus the micrograms of GSH in a Spectronic 20 or Spectronic 600 spectrophotometer.

Figure A-4-1 is a composite standard curve for GSH determined in this investigation by the method of Grunert and Phillips. The ordinate

is in optical density at 520 nm and the abscissa is graduated in micrograms of GSH. Each point on the curve is the average value of several routine determinations; the error bars represent one standard deviation. In this graph a straight line was fitted by eye through the origin and the average values; a statistical fit was not obtained.

The GSH values obtained in each group of determinations were obtained from the standard curve prepared for that day; each of these standard curves appeared to be linear and passed through the origin.

GSH Determinations

For determination of lenticular GSH, 0.4 ml of the TCA filtrate of the lens homogenate was allowed to stand at room temperature with 3.0 ml of SS NaCl. Then 1.6 ml of distilled water was added followed by additions of 0.5 ml each of NP and CN solutions. The mixture was capped with Parafilm, mixed by inversion and the resultant optical density determined at 520 nm on a Spectronic 20 or a Spectronic 600 spectrophotometer.

The quantity of GSH in the total lens (3.0 ml of lens homogenate) was determined by multiplying the quantity of GSH obtained from the above by 3.0 ml/0.4 ml. The concentration was then determined by dividing this latter value by the lens weight.

APPENDIX 5

OXIDIZED GLUTATHIONE (GSSG) DETERMINATION

Determination of GSSG was made by spectrophotometrically determining the decrement of $NADPH_2$ at 340 nm resulting from the reaction:

$$GSSG + NADPH_2 \xrightarrow[\text{reductase}]{\text{GSH}} 2\ GSH + NADP.$$

The amount of GSSG in the reaction is a function of the decreased amount of $NADPH_2$. (At 340 nm $NADPH_2$ absorbs strongly, whereas NADP does not.) The procedure used in this study was a modification of the procedure of Bergmeyer (1963).

Bergmeyer suggested determination of total GSSG in micrograms (μg) in the reaction by the formula:

$$GSSG = \frac{O.D_{340_{12min}} \text{ (reaction volume in ml) (612)}}{6.2} \qquad (1)$$

$\left[O.D_{340_{12min}} \right.$ is the decrement in optical density at 340 nm during the initial 12 minutes of reaction, 612 is the molecular weight of GSSG and 6.2 is the extinction coefficient (actually 6.2×10^6 cm^2/mol)$\left. \right]$.

In this investigation the method was modified in two ways: The $O.D_{340_{12min}}$ was plotted versus μG of GSSG to create a standard curve, and N-ethylmaleimide was added to the reaction to prevent oxidation of existing GSH which could result in a false high reading.

GSSG Standard Curve

The reagents requried for this assay were: (1) Buffer (B) consisting of M/10 phosphate buffer pH 7.6 plus 10^{-4} Sodium-ethylenediaminetetracetate (EDTA); (2) Albumin (ALB)—Bovine serum albumin 1% in Buffer (B); (3) Oxidized glutathione (GSSG) (Calbiochem Grade B crystals--lot 000567)—80 mg in 100 ml water diluted 1:10 with buffer (B). These solutions were made fresh prior to each series of determinations. (4) N-ethylmaleimide (NEM)--(Calbiochem B grade No. 34115) 15 mg—dissolved in 100 ml of Buffer (B). (5) $NADPH_2$ (tetra sodium salt of $NADPH_2$—Sigma) 10 mg in 2 ml of 5% sodium bicarbonate was maintained in light resistant capped glass vials—frozen between assays. (6) Glutathione reductase (Calbiochem Grade A lot 934093) 440 international units (IU) diluted to 60 IU with 3M urea. The enzyme was delivered to the reaction mixture from a 20 μL Lang Levy pipette.

The entire reaction was carried out in a standard quartz cuvette (1 cm light path).

Standard Curve

A blank was prepared as follows: 2.0 ml of Buffer (B), 0.15 ml of 1% albumin solution, 1.0 ml of GSSG solution, 0.1 ml of NEM and 20 lambda (μL) of diluted enzyme. The cuvette was capped with Parafilm and mixed by inversion. In determination of the OD decrement resulting from different quantities of GSSG, identical volumes were used as for the blank; however, 0.040 ml of $NADPH_2$ solution was added. 1 ml of GSSG solution contained 80 micrograms of GSSG. When lesser volumes of GSSG (e.g., equivalent to 40,20 or 10 μg) were used, the volume was adjusted to 1.0 ml with buffer (B). A standard curve was made by plotting the $OD_{340_{12min}}$ versus

μG of GSSG. This standard curve was compared with that obtained by the use of Bergmeyer's formula given above. The cuvettes were rinsed with concentrated nitric acid between determinations, then rinsed with glass distilled water--this removed all residual enzyme.

Figure A-5-1 shows the optical density as a function of time for various amounts of GSSG.

Figure A-5-2 is a standard curve for oxidized glutathione. The ordinate is graduated in units of OD 340/12 (the decrement of optical density at 340 during the initial 12 minutes of reaction), the abscissa, in micrograms of GSSG. The standard curve was quite linear and was reproducible when freshly diluted enzyme was used. For example, the resultant decrement in optical density during the initial 12 minutes of reaction versus micrograms of oxidized glutathione were 0.215 versus 80 μg; 0.108 versus 40 μg; and 0.058 versus 20 μg.

Values obtained several days following dilution of the enzyme were lower, but still quite linear (e.g., OD 340/12 versus μg GSSG: 0.180 versus 80; 0.091 versus 40; and 0.037 versus 20).

Bergmeyer stated that one could not utilize equation (1) for the determination of GSSG unless the reaction had gone to completion during the assay time interval. The slope of the reaction rate at 12 minutes as plotted in Figure A-5-2 indicated that the reactions were incomplete.

Two curves for OD 340/12 versus weight of GSSG are plotted in Figure A-5-3. The ordinate and abscissa are identical with those in Figure A-5-2 (OD 340/12 and micrograms GSSG, respectively). The lower curve is the standard curve for GSSG (Figure A-5-2). The upper curve resulted from plotting the OD 340/12 versus the weight of GSSG determined

by the use of the equation. The OD 340/12 versus the indicated weight of GSSG were 0.215 versus 66 μg; 0.108 versus 35 μg; and 0.058 versus 18.6 μg, respectively.

GSSG Determinations

The 0.5 ml aliquot of the TCA lens homogenate filtrate was neutralized with 0.15 ml of 1M sodium carbonate. The pH was checked with phydrion paper. The volume of the neutralized aliquot was adjusted to 3.0 ml with Buffer (B). 0.1 ml of NEM was added, then 0.15 ml of Alb 0.040 ml of $NADPH_2$ solution, and a final 0.020 ml of enzyme dilution. The cuvette was capped with a section of Parafilm, mixed by inversion and the initial optical density determined at 340 nm against the blank. The optical density was measured at 12 minutes. the GSSG was determined from curve A-5-2. The amount of GSSG in the lens was six times this value, as the original lens homogenate volume was 3 ml.

Reaction Rates of Known Quantities of GSSG

The reaction rates, as determined by the decrement of optical density at 340 nm (OD_{340}), were a function of substrate (GSSG) concentration. Figure A-5-1 is a plot of the reaction rates of 1.6 I.U. (International Units) of purified yeast glutathione reductase (GSH reductase) in the presence of 6×10^{-3}M $NADPH_2$, for various concentrations of GSSG. The concentrations of GSSG were 13,6.5 and 3.25×10^{-5}M (80,40 and 20 micrograms, respectively (Bergmeyer, 1963)). The ordinate is graduated in units of optical density at 340 nm; the abscissa, in minutes of reaction. The optical density appeared to have decreased in an exponential manner, and the curve was nearly asymptotic after 10 minutes of reaction. The

reaction was carried out for 12 minutes as specified by Bergmeyer. If the reaction were complete the slope of the curve should have been zero. The quantity of GSSG could have been then determined by the formula given above. The slopes of the reaction rates of GSSG did not appear to be zero after 10 to 12 minutes of reaction. They were, however, small.

Discussion of Assay

The enzymatic method of GSSG quantitation described by Bergmeyer (1963) was used in this investigation. One modification may have caused a decrement in the rate of GSSG reduction. This was the use of a smaller $NADPH_2$ concentration in the test assay. This was necessary in order that the initial optical density (O.D. at 340) be less than one. (The logarithmic scale for OD on the Spectronic 600 spectrophotometer used was not graduated sufficiently well to permit accurate readings in the ranges above OD 1.) A second modification was the addition of NEM (N,ethylmaleimide) for the removal of reduced glutathione. NEM combines irreversibly with sulfydryl groups. Sufficient NEM was added to each test reaction to remove all GSH present from the lens filtrates. (The GSH/aliquot was equivalent to 350 mg% wet weight of lens.)

The reaction rates of reduction of GSSG with glutathione reductase in the presence of $NADPH_2$ can be calculated from data used for the construction of Figure A-5-1. The methods utilized for the determination of rate constants and subsequently in the determination of half life are described by Frost and Pearson (1965).

Based on extrapolation of our data to zero reaction rate (in which the reaction rate did not appear to be complete at 12 minutes) it was difficult to determine that the reaction rate was indeed first order.

It is possible that if we had used a greater amount of $NADPH_2$ the reaction would have gone essentially to completion by 12 minutes, and calculations would have indicated a first order reaction. However, as indicated above, it was not possible to use a greater amount of $NADPH_2$ because of spectrophotometric limitations.

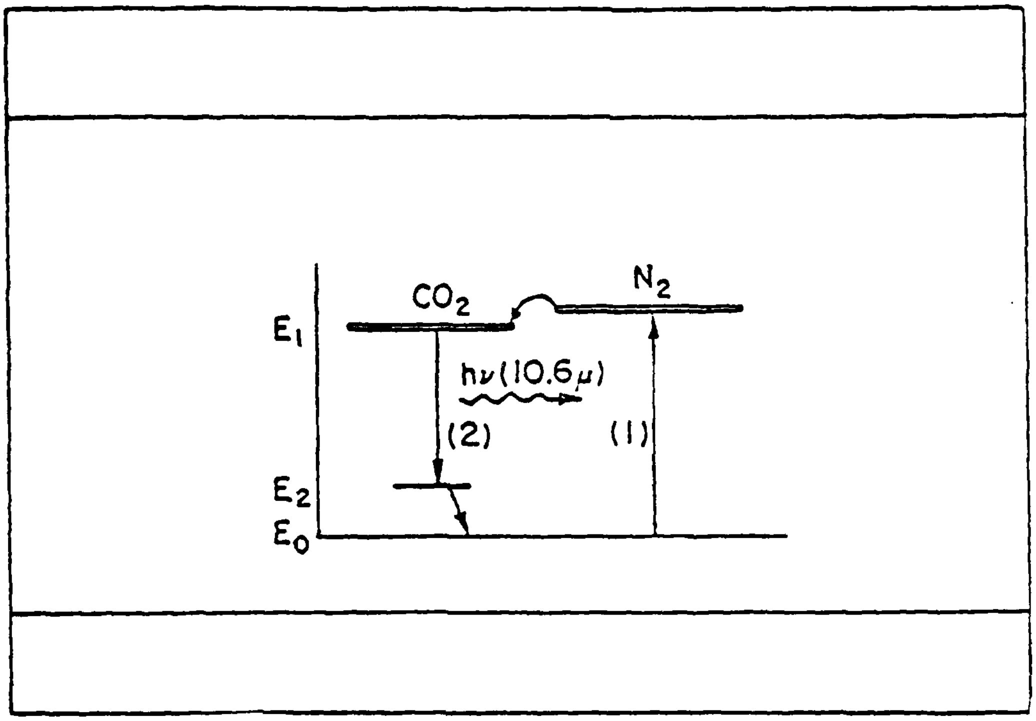


Figure In-1 The first step in population inversion process shown in a schematic simplified fashion for a CO_2 laser system. The numbers in brackets indicate the sequence of events. Step 1, on excitation, the nitrogen molecule (N_2) is elevated in energy from the ground state E_0 to a discrete level E_1. This level is similar to the E_1 level of CO_2 and the energy is transferred to the CO_2 molecule. In Step 2, the energy of the CO_2 molecule relaxes to a level E_2 and a photon of energy $h\nu$ equal in energy to $E_1 - E_2$ is emitted at 10.6μ. When more CO_2 molecules are at a level E_1 than at E_2, the situation is termed a population inversion.

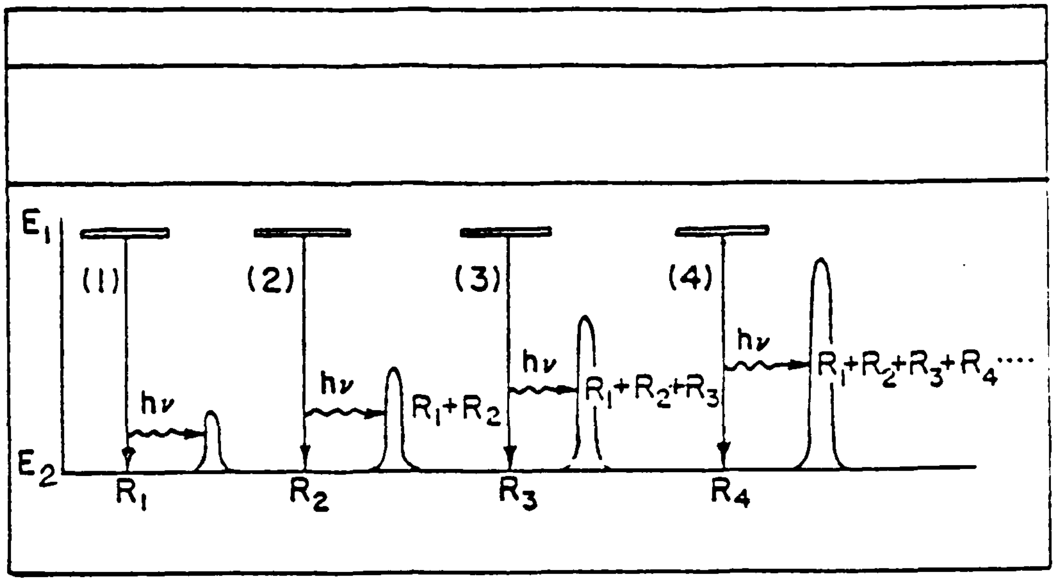


Figure In-2 A simplified schematic representation of stimulated emission of radiation. The sequence of events from left to right is indicated by the numbers in brackets. Many CO_2 molecules have been raised to the excited state E_1. In Step I the energy of one molecule relaxes from E_1 to E_2 spontaneously. The emitted photon hν is absorbed by an adjacent molecule which is at energy level E_1. This collision results in a decrease of energy in the second molecule from E_1 to E_2 and the release of a second photon at 10.6μ. These two photons are identical in wave length and are in phase. The process continues through R_2, R_3 and R_4. The output, therefore, consits of photons which are all essentially at 10.6μ and in phase. The radiation of the first molecule is, therefore, amplified due to stimulated emission of radiation from the molecules at 2, 3, and 4.

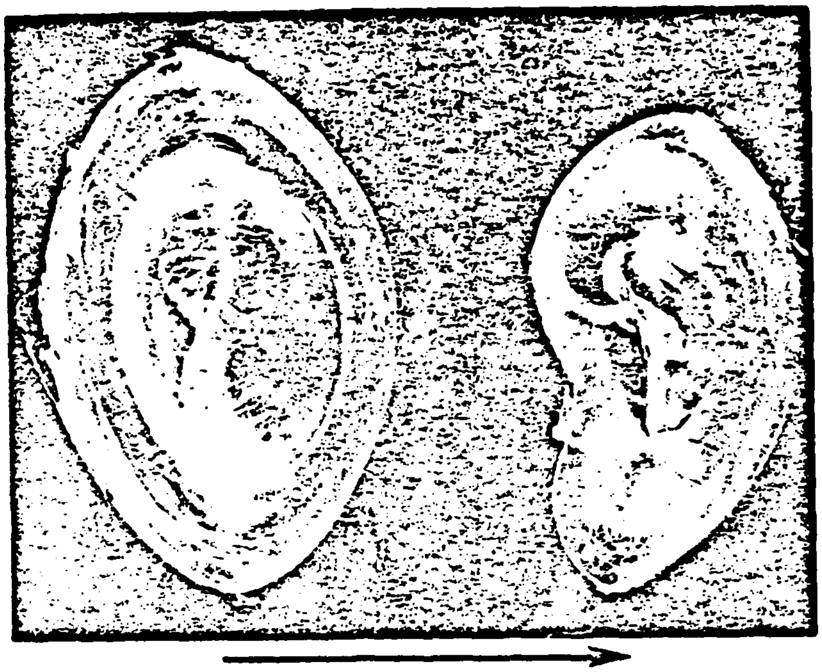


Figure I-1 shows the appearance of a lens cross-section from a control and from a CO_2 laser irradiated eye respectively, in which the cornea had not been perforated. Both the lenses were removed from eyes fixed in glutaraldehyde. The lens on the left taken from an unirradiated eye had a normal biconvex appearance. In contrast the anterior portion of the lens from the irradiated unpenetrated eye had a marked concavity. The free arrow shows the direction of the irradiating beam with respect to the lens.

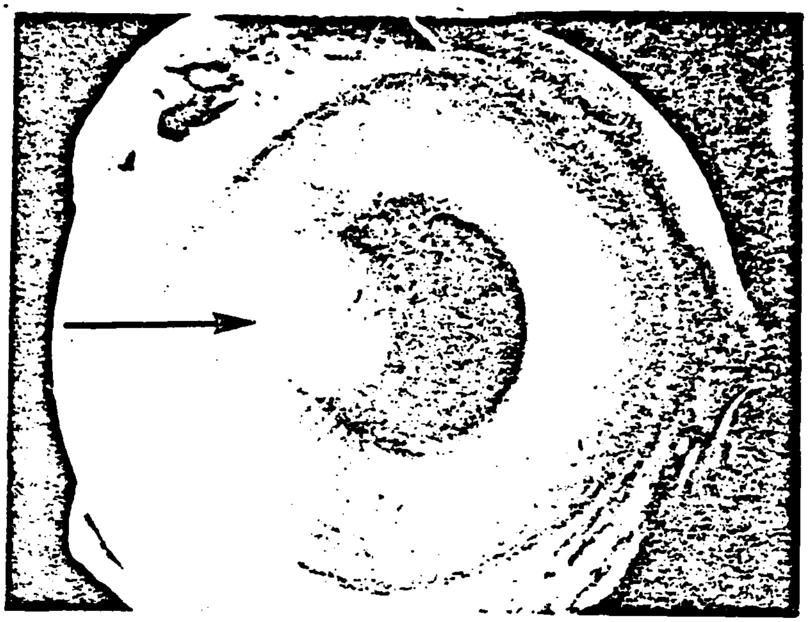


Figure I-2D

Figure I-2S

Figures I-2D and I-2S show the clinical appearance of the enucleated right and left eyes of a rabbit months after CO_2 laser irradiation. An arrow marks the periphery of the whitened scarred areas of each cornea. Subsequent histological examination (Fig. I-3D) shows that the upper cornea in Figure I-2D had perforated.

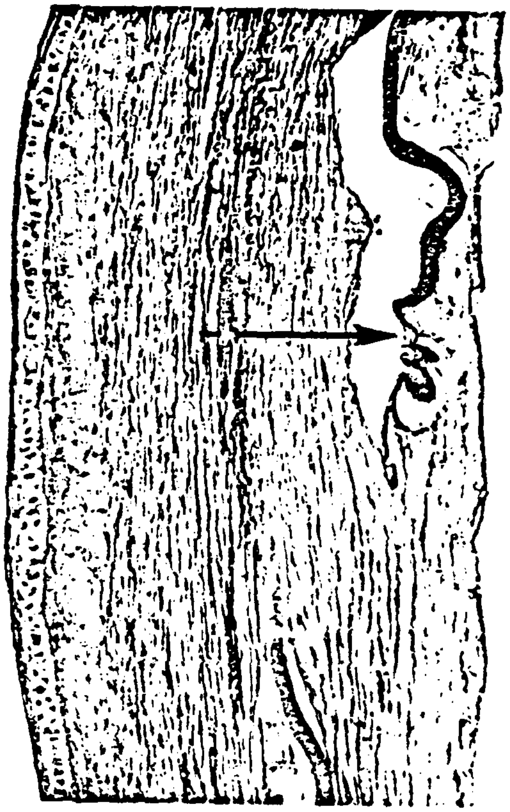


Figure I-3D Histological appearance of the irradiated region of a perforated rabbit cornea shown in Figure I-2D. The free arrow shows both the direction of the irradiating CO_2 laser beam as well as the curled end of Descemet's membrane. The curled end indicates that Descemet's membrance has been ruptured and, therefore, that this cornea had perforated (X160 PAS stain).

Figure 1-4D

Figure 1-4S

Figures I-4D and I-4S The appearance of lenses by retro-illumination. The upper lens (I-4D) was taken from an eye whose cornea had perforated on irradiation with the CO_2 laser. The upper lens shows positive cataractous change. The free arrows are directed to some of the cloudy regions. These changes were not apparent in the lens of the other eye (Figure I-4S). The black lines and specks in I-4S are artifacts.

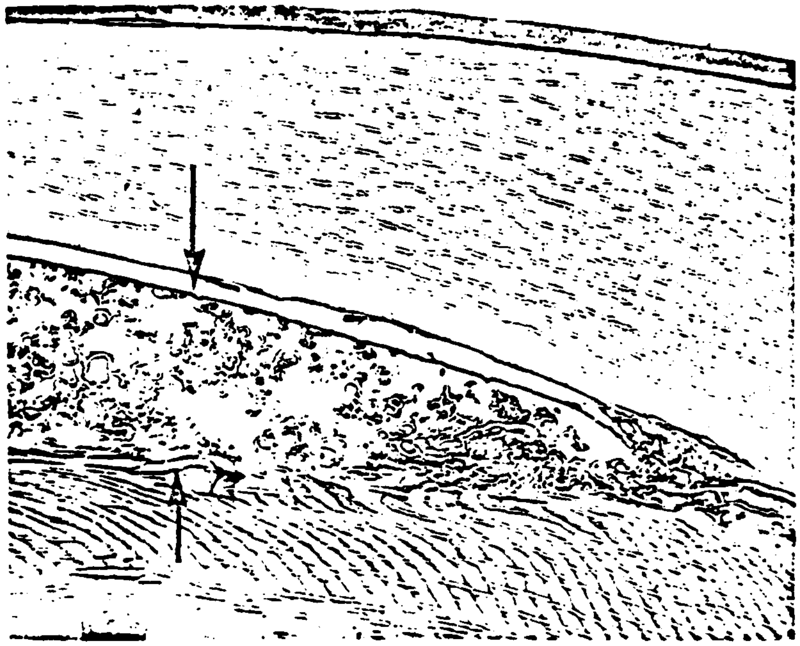


Figure I-5D

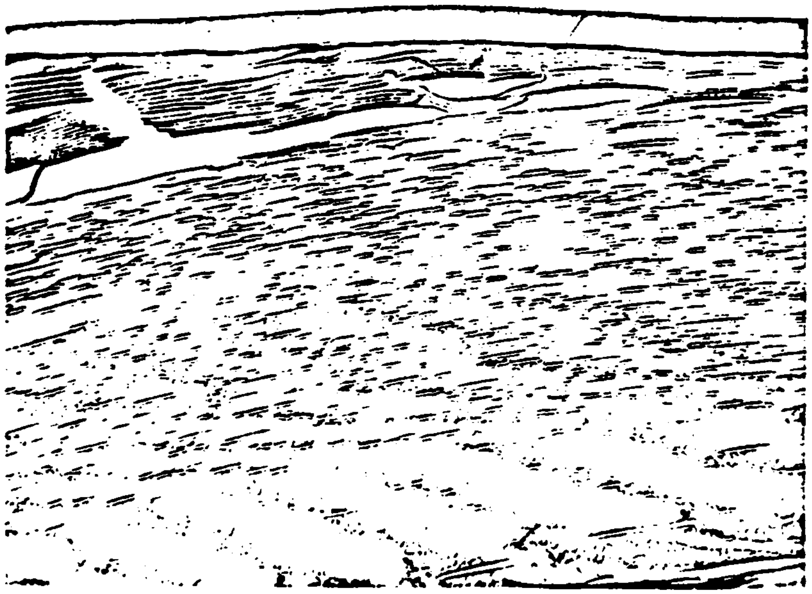


Figure I-5S

Figures I-5D and I-5S Histological appearance of the anterior region of lenses excised from eyes (perforated and unperforated, respectively) on CO_2 laser irradiation. The upper figure shows a region (between the arrows) of cataractous changes deep to the anterior lens capsule, in an eye in which the cornea has been perforated. The lower figure (I-5S) shows the appearance of a comparable region of the lens from the unperforated left eye—no cataractous changes are present. The clefts in both sections are artifacts (X140 H and E).

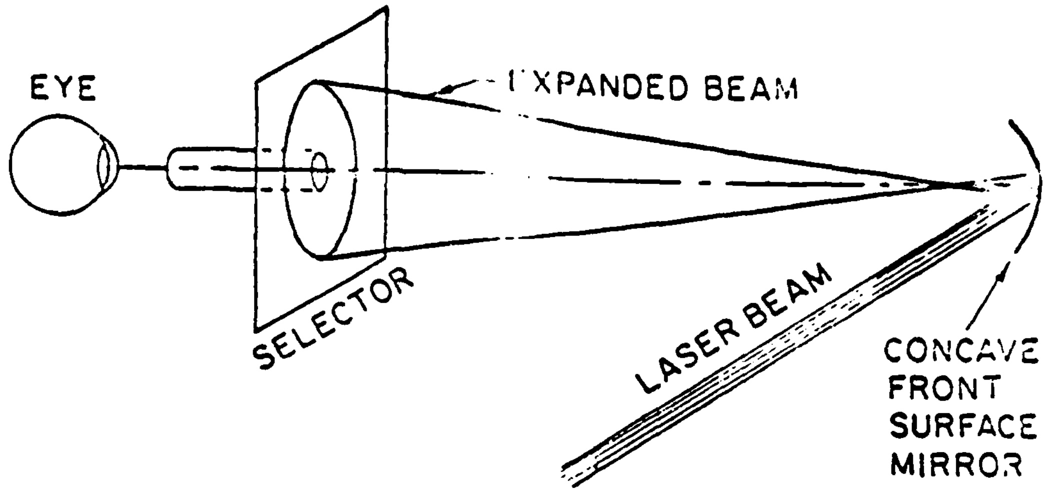


Figure II-1 A diagrammatic representation of the set-up employed in this study to irradiate the cornea with a selected portion of the CO_2 laser beam reflected from a concave, front surface mirror.

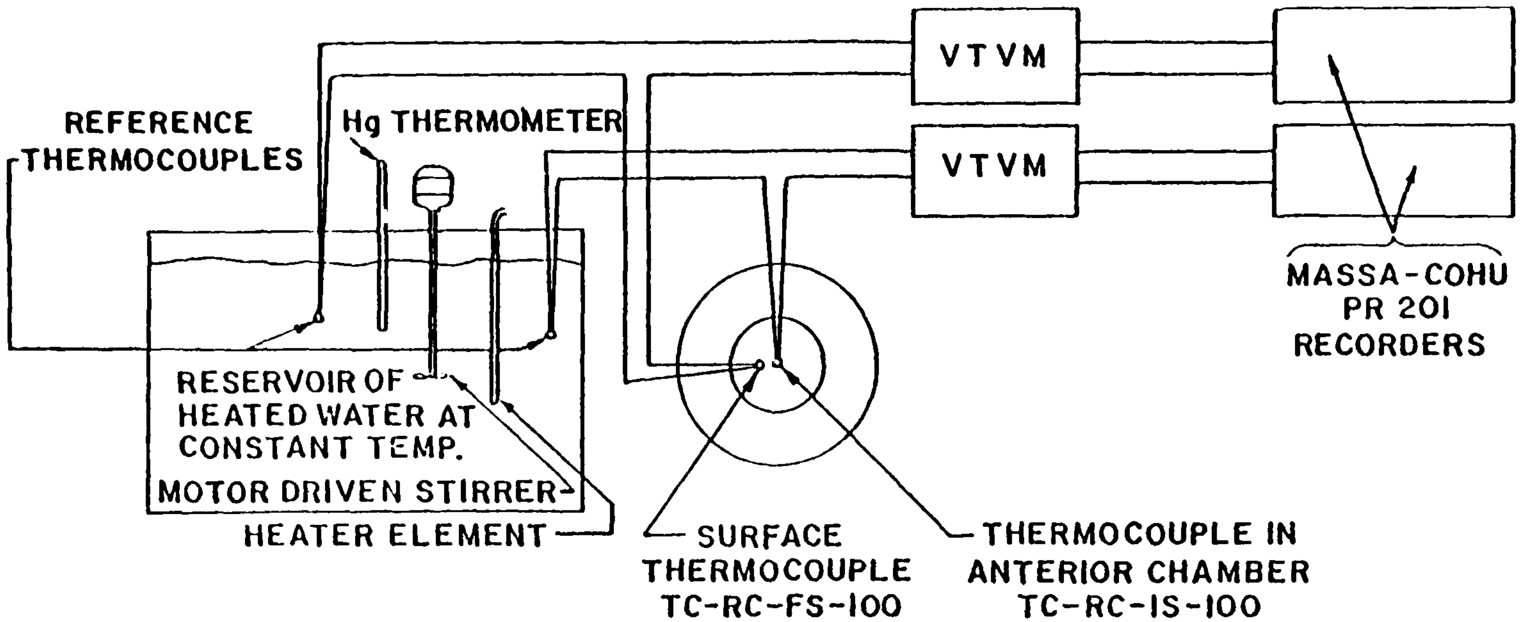


Figure II-2 Simplified representation of the system employed in this study for simultaneous recording of corneal surface and anterior chamber temperatures by means of separate thermocouples.

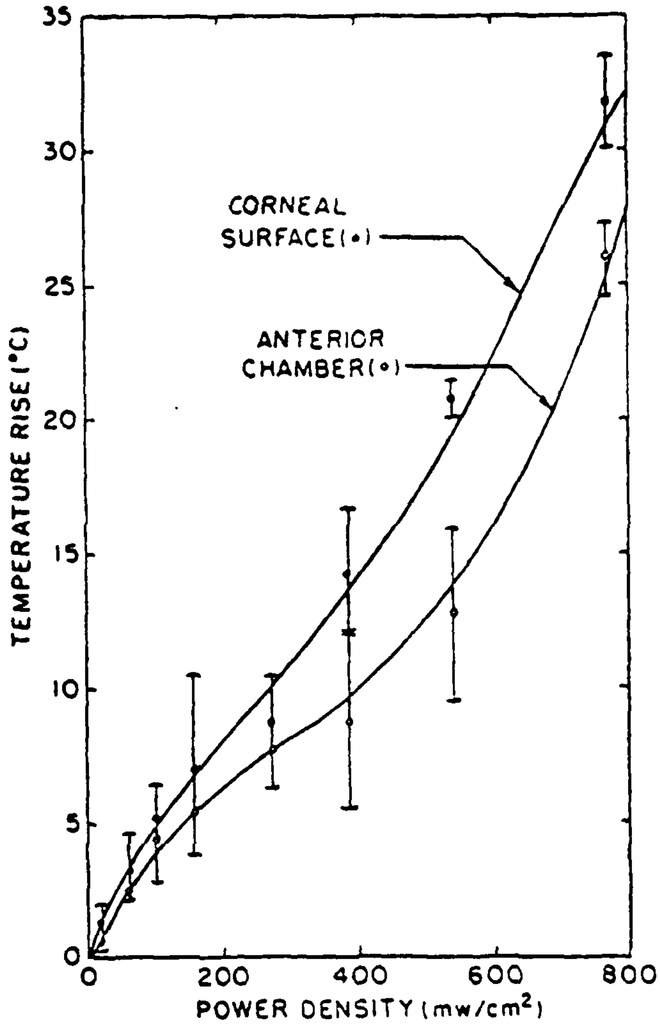


Figure II-3 Steady state corneal surface and anterior chamber temperature elevations during CO_2 laser irradiation of the rabbit cornea. Each circle is the average value for various irradiating power densities. The error bars represent one standard deviation.

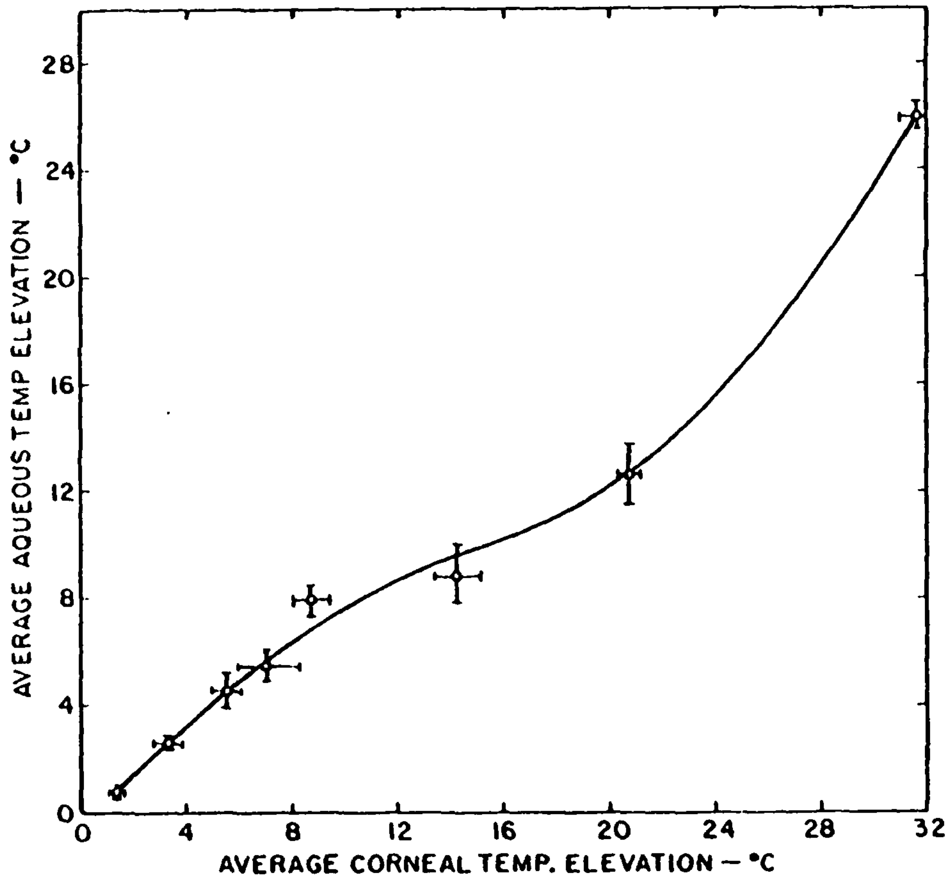


Figure II-4 Steady state elevation of aqueous humor temperature as a function of corneal surface temperature during continuous CO_2 laser irradiation of the cornea of the rabbit (20 to 770 mw/cm^2). Each circle is the average value; the error bars represent one standard deviation.

mW/cm^2	Cornea ΔT in °C	Aqueous ΔT in °C
20	1.3 ± 0.95(3)	0.62 ± 0.88(3)
60	3.3 ± 2.33(3)	2.5 ± 0.32(3)
100-110	5.5 ± 2.3(5)	4.4 ± 2.9(5)
155	7 ± 5.3(2)	5.5 ± 2.4(2)
270	8.7 ± 2.7(2)	7.9 ± 2.3(2)
390	14.25 ± 3.36(3)	8.75 ± 4.6(3)
580	20.8 ± 1.2(3)	12.7 ± 4.81(3)
770	31.7 ± 2.5(2)	26. ± 2.0(2)

Table II-1 Steady state temperature elevation at corneal surface and in aqueous during CO_2 laser irradiation of the eye, at various power density levels. ± one standard deviation. The parentheses indicate the number of eyes used for each power density determination.

Power Density (mW/cm^2)	Cornea		Mid Chamber	Lens Surface	Iris	
	Ant.	Post.			On	Under
270	42°C	41.5°C	40.5°C	40°C	39°C	38.5°C
340	52°C	50°C	-	48°C	48°C	46°C

Table II-2 Steady state temperatures measured on the cornea and in the anterior segment of the rabbit eye during continuous CO_2 laser irradiation of the cornea.

	Cornea	Anterior Chamber
This investigation*	32.3 ± 1.43°C	34.4 ± 1.8°C
Schwartz & Feller(1962)	32.3 ± 0.49°C	32.98 ± 0.74(mid) 33.60 ± 1.01(post)

Table II-3 Average corneal and anterior chamber temperatures of unirradiated rabbit eyes at room temperature, compared with data from Schwartz and Feller. The asterisk represents the average of 24 eyes.

Power Density (mW/cm^2)	Dry*	Wet*
1000	14.8°C	8.5°C
500	7.4°C	1.94°C
250	5.45°C	1.17°C

Table II-4 Temperatures measured on direct CO_2 laser irradiation of a chromel—constantan thermocouple in air. The asterisk represents the average value for each determination. The reference junction was in water at 22°C. The air temperature was 22°C.

Present Investigation		Hoffmann and Kunz(1934)	
Corneal	Aqueous Humor	Corneal	Aqueous Humor
47°C	43.6°C*	47-48°C	42°C
50°C	45.4°C*	50-51°C	46-47°C

Table II-5 Comparison of aqueous humor temperature* obtained in this study with those of Hoffmann and Kunz (1934) for similar corneal temperatures. The asterisk represents the values of aqueous humor temperature shown for the present investigation were obtained from the data shown in Table II-3 and Figure II-3. The initial corneal temperature in this study was 32.3°C, the aqueous humor temperature was 34.4°C (Table II-3).

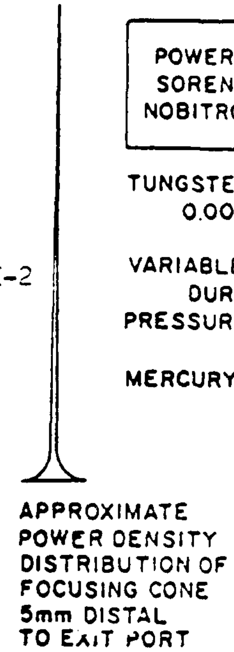


Fig. III-2

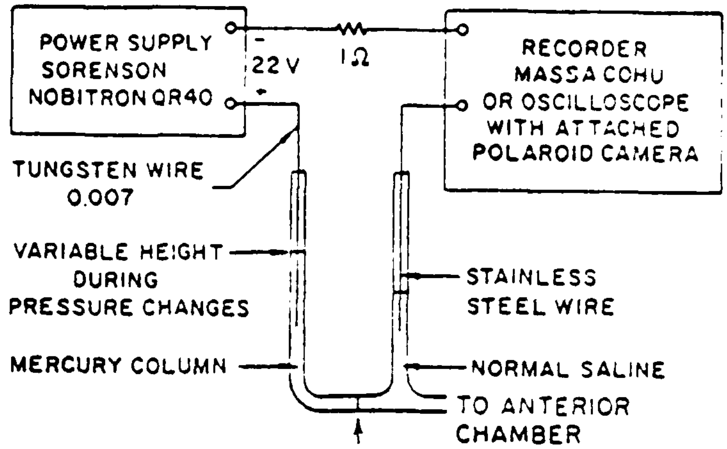


Fig. III-4

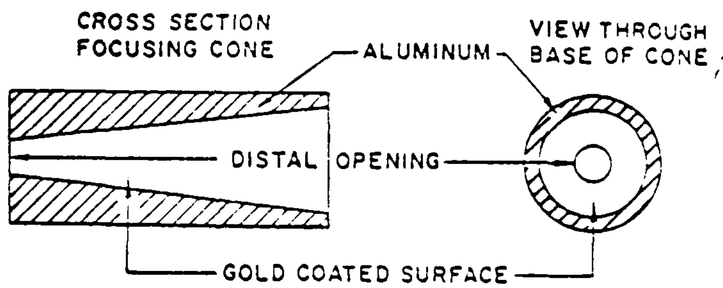


Fig. III-1

Figure III-1 Cross-sectional view of the cone used in this study to focus the CO_2 laser output. The diameter of the distal opening (output port) is 5 millimeters.

Figure III-2 A sketch of the expected power density distribution in a region approximately 5 millimeters distal to the exit port of the focusing cone. The height roughly corresponds to the relative estimated power density; the width of the base corresponds to the dimensions of the exit port. The power density is markedly highest at the center. This agreed with attempted thermocouple measurements and Thermo-fax paper patterns.

Figure III-4 A simplified diagrammatic representation of the circuitry used for electrical recording of intraocular pressure variations with the mercury manometer.

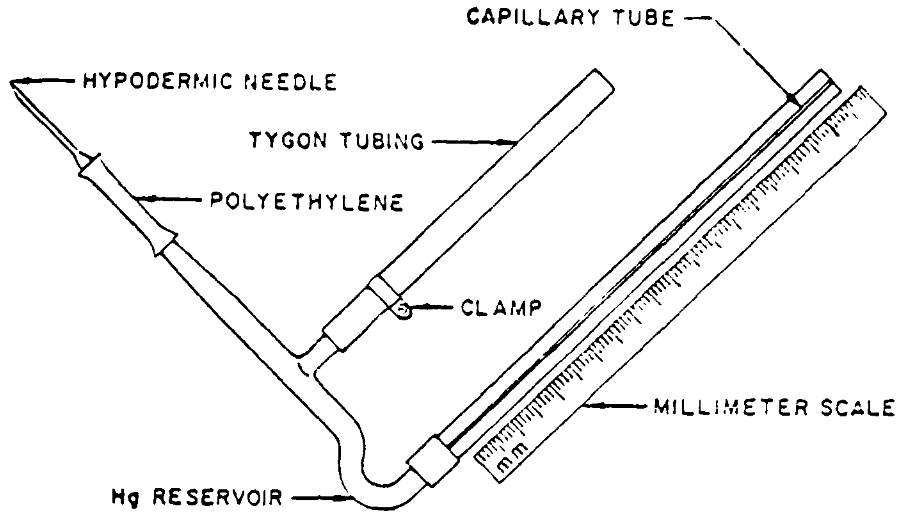


Figure III-3 Schematic representation of the mercury manometer system used in the preliminary investigation of intraocular pressure measurements during CO_2 laser irradiation of the corneal surface.

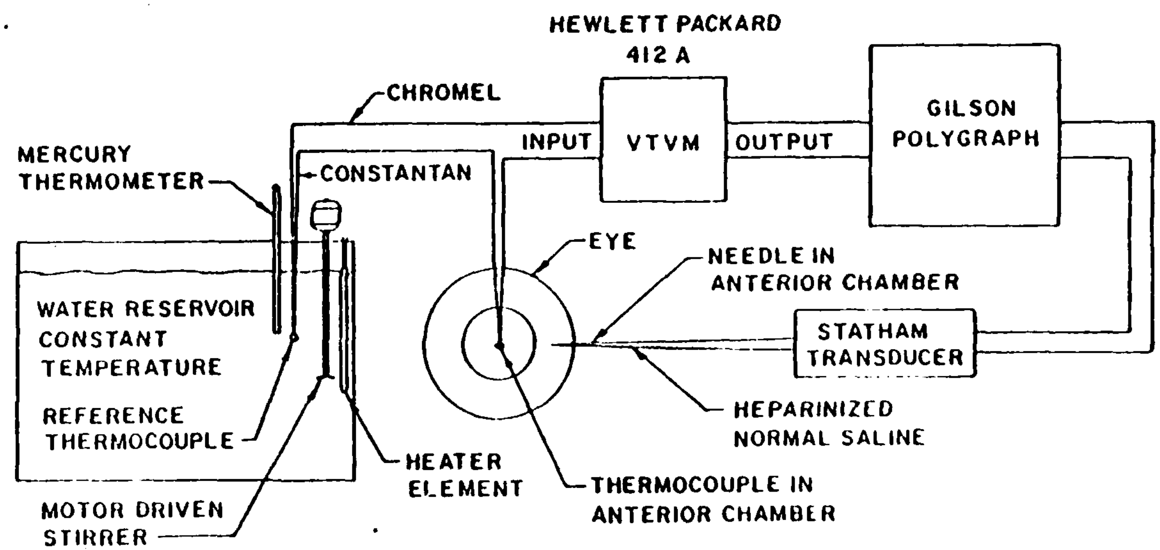


Figure III-5 A schematic representation of the equipment employed in this study to record simultaneous temperature and pressure changes in the anterior chamber of the rabbit eye during CO_2 laser irradiation of the cornea.

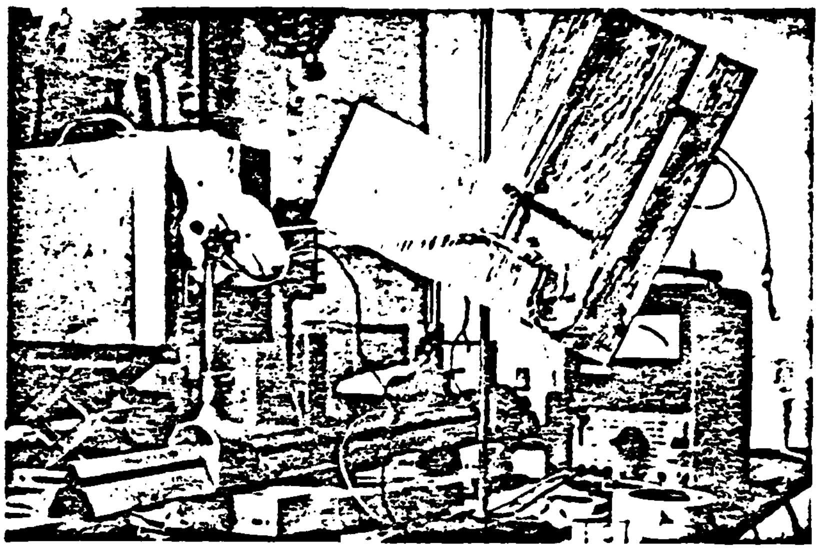


Figure III-6 A view of an anesthetized rabbit positioned for determination of intraocular pressure changes during cone-focused CO_2 laser radiation on the mid-anterior surface of the cornea. The manometer-system needle is sealed into the anterior chamber. The Hg manometer depicted in Figure III-3 is shown at the right; the focusing cone diagrammed in Figure III-1 is shown at the left.

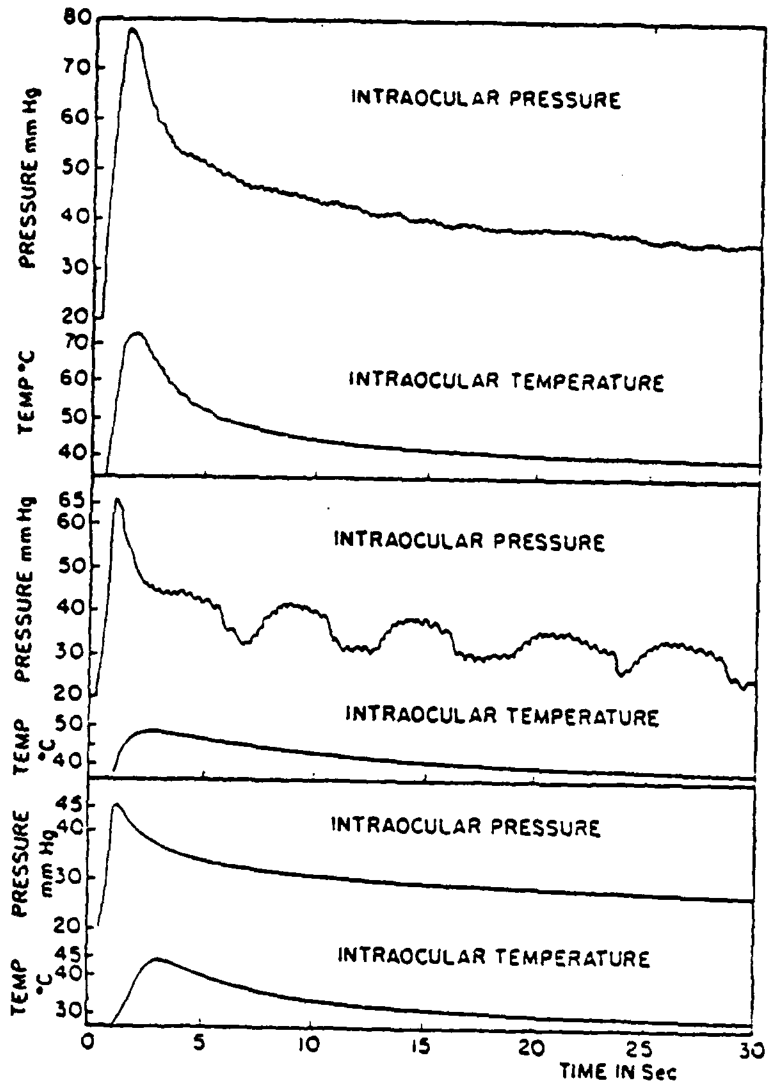


Figure III-7 Simultaneously recorded pressure and temperature variations measured in the anterior chambers of rabbit eyes during focused CO_2 laser irradiation of the mid-corneal surface. The initial intra-ocular pressure at zero time was 20 mmHg—the initial point of each pressure trace is at this value. The top pair of traces (pressure and temperature) are for 1.5 second irradiations at 6 watts, the middle and lower pair of traces are for 0.75 second irradiations at 6 watts. Each irradiation started at zero time. The upper and middle pairs of recordings were made in living animals. In the bottom graph, the animal had died prior to irradiation. Respiration and blood pressure variations present in the upper two graphs are absent from the bottom graph.

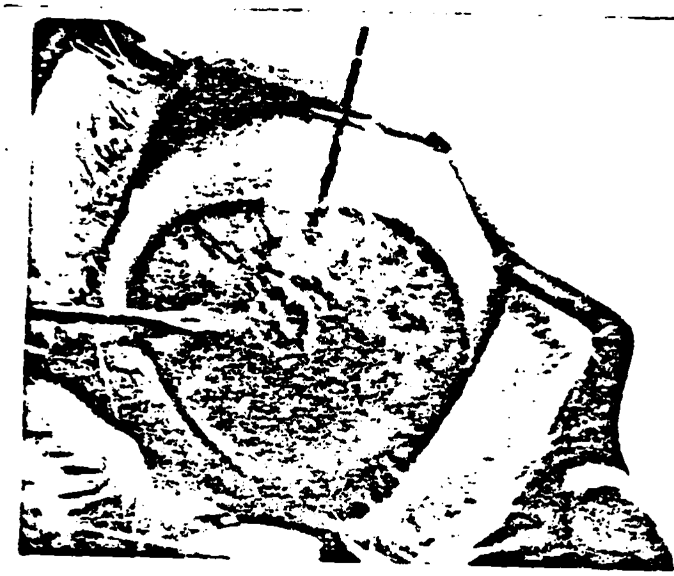


Figure III-8 View of a rabbit eye immediately following focused CO_2 laser irradiation (6 watts, 1.5 seconds). The transducer system needle (9 o'clock) and the thermocouple (12 o'clock) are sealed into the anterior chamber. The site of irradiation in the approximate center of the cornea is visible.

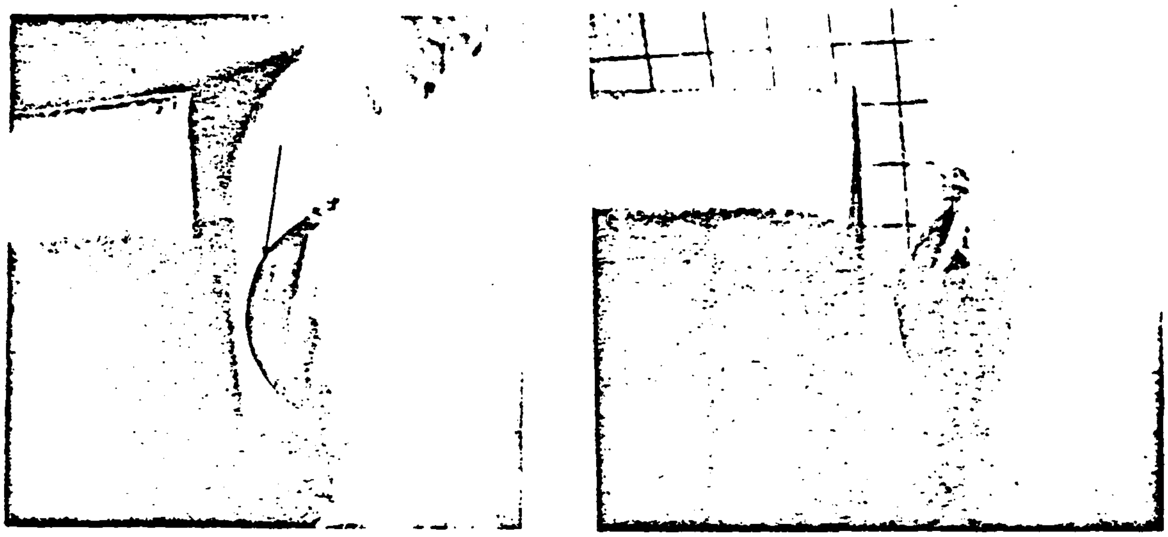


Figure III-9 Lateral views of a rabbit cornea before and immediately following focused irradiation of the mid-corneal surface with a CO_2 laser (7 watts, 1 second). A portion of the focusing cone is visible in each view. In the figure at the left, taken prior to irradiation, there is a definite space (arrow) between the cornea and the anterior surface of the lens. This space is not visible in the figure at the right taken immediately following irradiation; the radius of curvature of the corneal surface is greater following irradiation—the cornea appeared flattened.

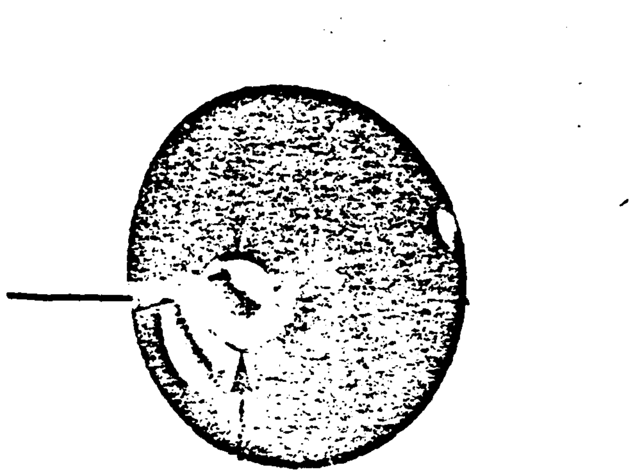
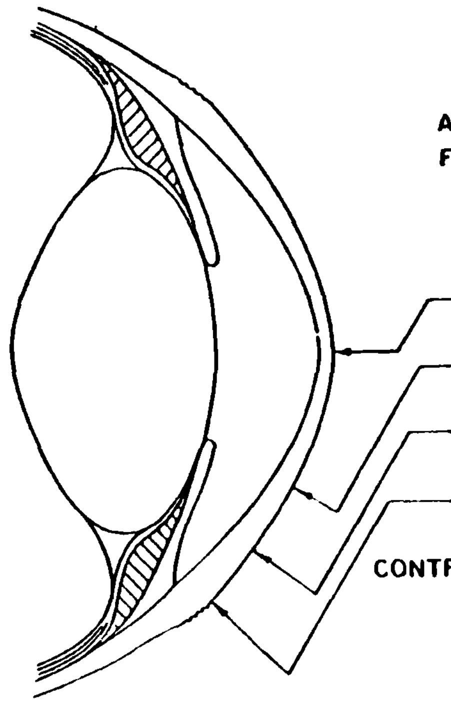


Figure III-10 A concavity in a fixed lens following CO_2 laser irradiation (6 watts, 1.5 seconds) of a rabbit cornea. The lens was stained by immersion in acid orcein following dissection from an excised eye fixed in glutaraldehyde. The lens is shown at an oblique view. The concavity in the center of the anterior surface of the lens is located between the arrows. The entire surface of the lens was darkly stained except for the region of the concavity. The extent of this lessened staining is partially visible as the cloudy-white region between the two horizontal and bottom arrows. The white areas outside the arrows and that within the arrows at 1 o'clock represent reflections and are photographic artifacts.



AQUEOUS HUMOR PROTEIN LEVEL (mg/100cc) FOLLOWING CO_2 LASAR IRRADIATION

	0+HOUR	24 HOURS	48 HOURS
A	60	60	60
B	123	296	350
C	183	636	543
D	76	102	—
CONTROL		—60—	

Figure III-11 Each eye was irradiated and sampled only once for aqueous humor protein. The sites of irradiation of the cornea were: (A) mid-pupillary, (B) edge of iris, (C) mid-iris, and (D) ciliary body region.

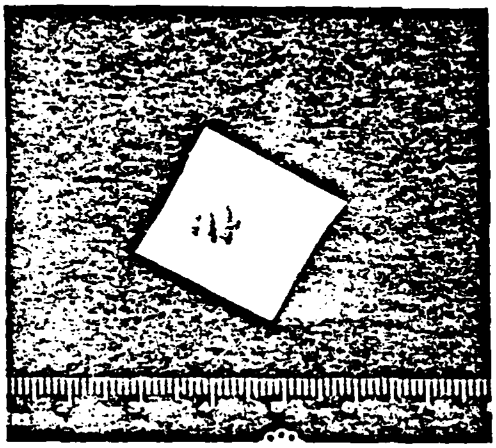


Figure IV-1 Pattern of darkening of Thermofax paper caused by the CO_2 laser beam in a multimode condition

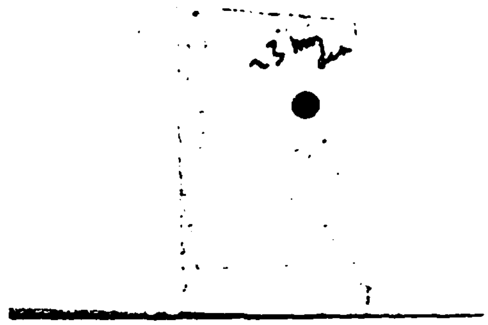


Figure IV-2 Pattern produced in Thermofax paper by a CO_2 laser beam assumed to be operating in a TEM_{oo} mode.

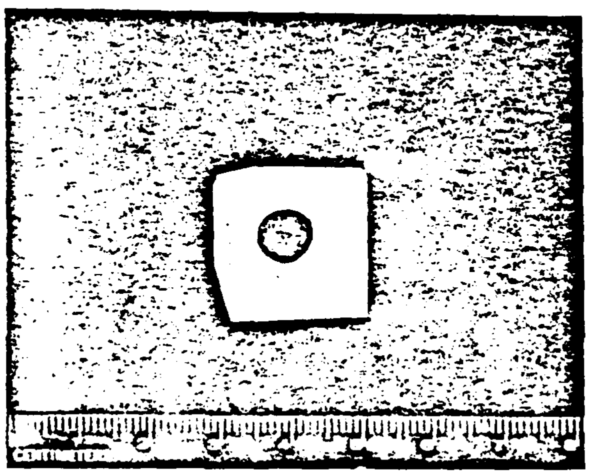


Figure IV-4 The pattern produced on Thermofax paper by the expanded CO_2 laser beam after passage through the 8 millimeter diameter aperture. The central area is darker than the peripheral zone. There appears to be uniform darkening radially.

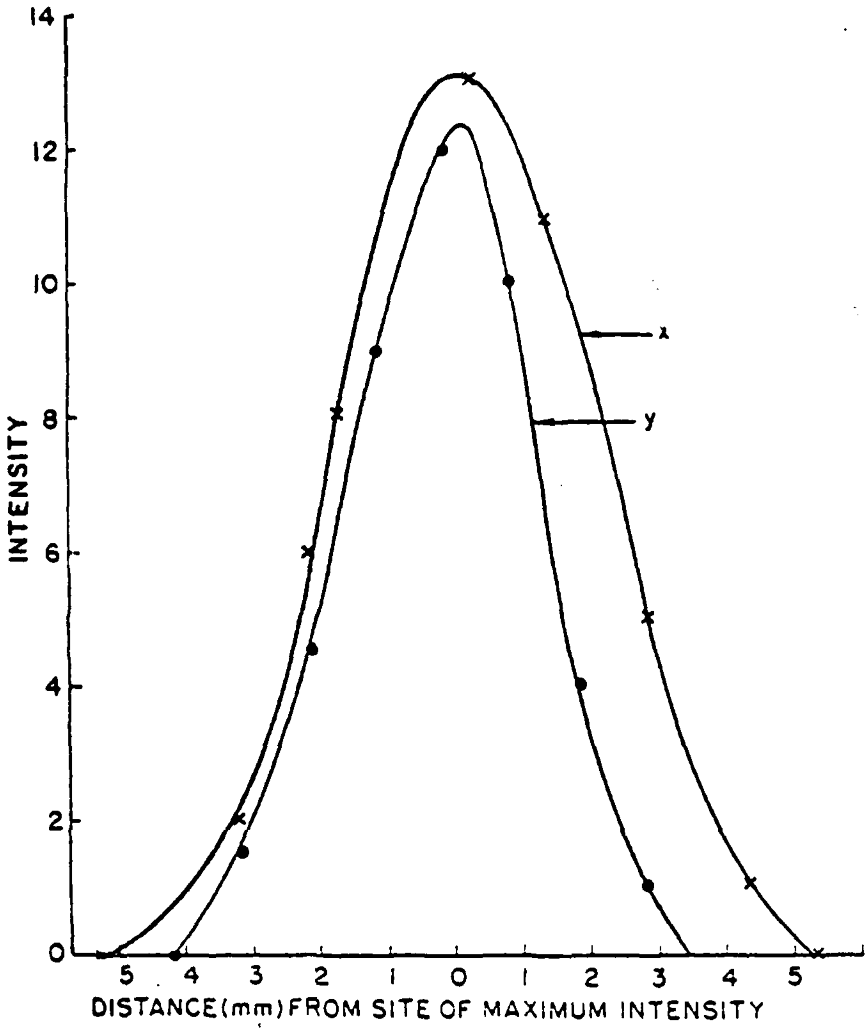


Figure IV-3 Relative power density obtained on scanning a CO_2 laser beam in the X and Y directions. The pattern produced by the beam on Thermofax paper is shown in Figure IV-4.

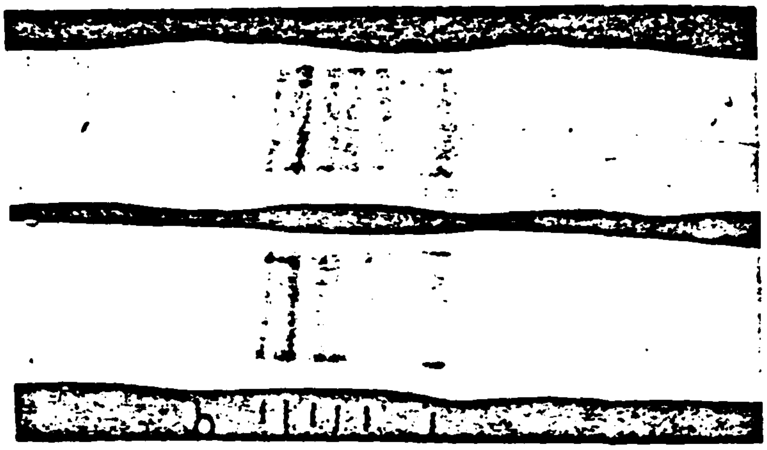


Figure IV-5 The electrophoretic pattern on cellulose polyacetate strips of soluble lenticular proteins from a pair of lenses from unirradiated rabbit eyes. The sample was applied at "O". The direction of migration was from left to right towards the anode. Six separate bands with identical mobilities, visible on each strip, are delineated by the lines beneath the lower strip. (Actual size)

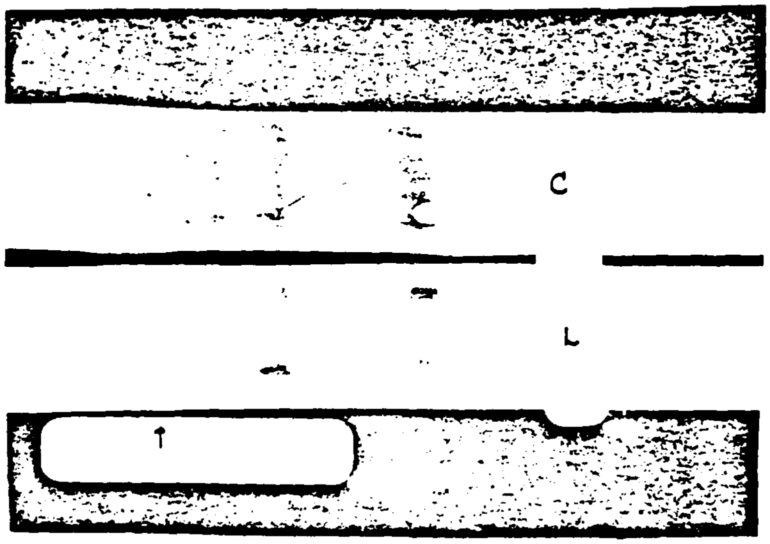


Figure IV-6 The electrophoretic pattern produced on cellulose polyacetate strips from an eye excised immediately following CO_2 laser irradiation of an 8 mm diameter area of the cornea (1.5 watts, 90 seconds). The strip labelled "L" is from a lasered eye; the strip labelled "C" is from the contralateral control eye. The number of bands and their mobilities appear to be identical. The electrophoretic strips appeared to have six bands—this number of bands may not be evident in this photograph (Actual size)

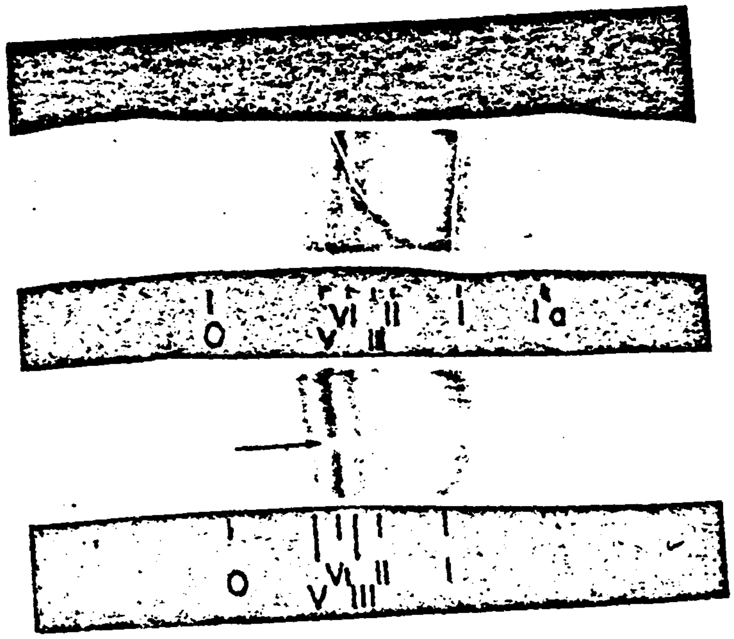


Figure IV-7 The electrophoretic separation of lenticular proteins from an eye (lower strip) excised three days following CO_2 laser irradiation (1.5 watts, 90 seconds) of the cornea compared with that from the contralateral control eye. The mobilities of the protein groups on the cellulose polyacetate strips are not identical. The mobilities of the slowest bands (arrow) in the lower strip were less than that of the control. The mobilities of the other bands do not appear to have been remarkably altered. The fast band, Ia, is present only in the control strip. (Strips actual size.)

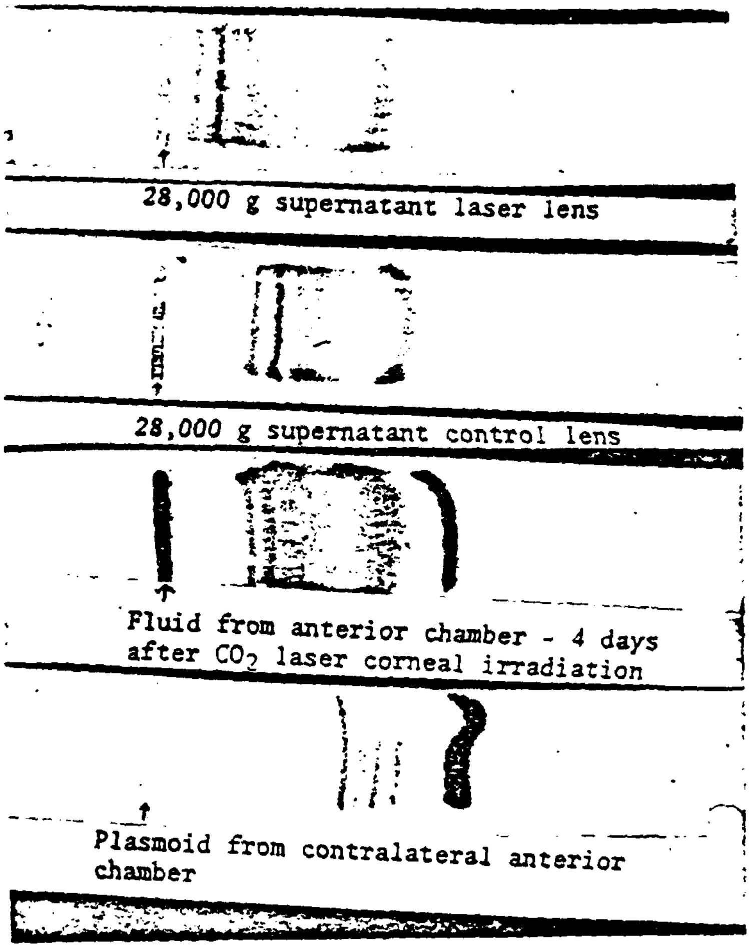


Figure IV-8 The electrophoretic pattern of lenticular and aqueous humor proteins from an eye four days following CO_2 laser irradiation (1.5 watts, 90 seconds, 3 mm diameter area) compared with that of the contralateral control eye. (Previous paracentesis had been performed on the anterior chamber of the control eye to cause the formation of plasmoid aqueous with a high protein concentration in order that the bands be visible.) The mobility of the bands produced by the soluble proteins from the lens of the irradiated eye was comparatively less than that from the lens of the control eye. The fastest band produced by the plasmoid aqueous (bottom strip) was greater than that of either control or irradiated lenticular material; also the mobility of the slowest band was greater than that of the slowest band of the lenticular protein. The pattern produced by the aqueous humor protein from the irradiated eye (third strip) appears to be a composite of both control lenticular and control aqueous humor proteins. The arrows show the site of application of the samples.

TABLE IV-1

THE EFFECT OF CO_2 LASER IRRADIATION OF THE CORNEA* ON THE APPEARANCE OF THE LENS, THE ELECTROPHORETIC PATTERN, WEIGHT, ASCORBIC ACID (AsA) AND REDUCED GLUTATHIONE (GSH) OF THE LENS AND THE AsA IN AQUEOUS HUMOR

No.	Time after exposure	Eye	Lenticular Appearance	Lenticular Electrophoretic pattern	LENS (mg) weight	LENS % control	LENS AsA conc. mg%	LENS % control	LENS GSH conc. mg%	LENS % control	AQUEOUS HUMOR AsA conc. mg%	AQUEOUS HUMOR AsA % control
856	—	S	CLEAR	FIVE TO SIX SEPARATE BANDS, EQUAL IN MOBILITY AND WIDTH IN PAIRS OF LENSES.	218.9	103	13.8	106	300	93.7	10.4	82.5
	—	D			215.3		13.		320		12.6	
963	—	S			212.8	100	12.7	94.7	254	100	19.6	110
	—	D			213.3		13.4		254		17.8	
1330	—	S			224.8	84.3	16.1	94.1	290	111	24.8	124
	—	D			266.5		17.1		259		20.8	
826	—	S			211.4	99	11.1	95	373	100	10.5	81
	—	D			212.1		11.7		372		13.	

LEGEND: (*) 0.8 centimeter diameter of left eye radiated at 1.5 watts for 90 seconds. The mobilities of the electrophoretically separated bands of 2800 × g supernatant of lens homogenate (3 ml) are compared with corresponding bands of contralateral control lens supernatant. Other lenses were assayed for ascorbic acid and glutathione. The dashes in the right hand sections mean that data was not obtained.

TABLE IV-1 (continued)

No.	Time after exposure	Eye	Lenticular Appearance	Lenticular Electrophoretic pattern	LENS (mg) weight	% control	LENS AsA conc. mg%	% control	LENS GSH conc. mg%	% control	AQUEOUS HUMOR AsA conc. mg%	AQUEOUS HUMOR AsA % control
1338	—	S			203.2	107	12.5	108	244	94	31.3	124
	—	D			189.8		11.6		280		25.5	
26A	2 hours	S*	FAINTLY CLOUDY	FIVE TO SIX IDENTICAL BANDS--NO VISIBLE CHANGE FROM THAT OF UNIRRADIATED EYES	140.0	74	11.2	81	160	58	—	—
		D			190.1		13.8		276		19.5	
26B	2 hours	S*			172.1	91	14.5	93	305	128	5.	39
		D			189.4		15.5		237		12.9	
31A	1 day	S*			202.3	111	19.9	115	243	119	16.5	68
		D			181.5		17.3		204		24.4	
32B	1 day	S*			221.9	102	13.7	93	296	91	11.2	57
		D			217.3		14.7		326		19.6	
33D	1 day	S*			204.3	98	9.5	76	320	89	5.3	37
		D			209.1		12.6		359		19.4	
36G	3 days	S*	DESCRIPTION ON NEXT PAGE		172.5	73	6.6	42	96	29	—	—
		D			237.7		15.8		335		18.6	

TABLE IV-1 (continued)

No.	Time after exposure	Eye	Lenticular Appearance	Lenticular Electrophoretic pattern	LENS (mg) weight	% control	AsA conc. mg%	% control	GSH conc. mg%	% control	AQUEOUS HUMOR AsA conc. mg%	% control
38N	3 days	S*	LIQUID REGION IN ANTERIOR REGION OF CORTEX OF LENS; SL. CLOUDY LENS.	FIVE TO SIX BANDS. MOBILITY OF β AND γ CRYSTALLINS ABOUT 80% OF NORMAL; α CRYSTALLIN MOBILITY SLIGHTLY LESS THAN IN CONTROL LENS.	156.2	83	9.4	55	188	59	—	—
		D			189.1		18.8		317		14.	
39F	3 days	S*			253.5	112	6.3	65	130	53	—	—
		D			226.4		14.6		245		15.3	
40E	3 days	S*		CAPSULE SPONTANEOUSLY SEPARATES FROM LENS.	—	—	—	—	—	—	—	—
		D			269.4		18.1		300		23.	
44H	4 days	S*	VOLUME OF CORTICAL LIQUEFICATION INCREASED SUPERIOR REGION OF POST-ERIOR WHITENED.	MOBILITY OF β AND γ CRYSTALLIN BANDSGREATLY DECREASED—~50% OF NORMAL. α CRYSTALLIN MOBILITY APPROX. 85% THAT OF CONTROL LENS.	198.6	96	8.8	106	139	49	0.4	2.7
		D			207.5		8.2		286		17.6	
64D	5 days	S*			208.6	96	4.	24	140	29	1.4	12
		D			217.		17.5		480		11.4	
64A	5 days	S*			187.6	97	7.	50	230	39	3.3	35
		D			194.3		14.1		590		9.4	

TABLE IV-1 (continued)

No.	Time after exposure	Eye	Lenticular Appearance	Lenticular Electrophoretic pattern	LENS (mg) weight	LENS % control	LENS AsA conc. mg%	LENS % control	LENS GSH conc. mg%	LENS % control	AQUEOUS HUMOR AsA conc. mg%	AQUEOUS HUMOR AsA % control
64B	5 days	S*	REMAINING LENS MATERIAL WHITENED, AMORPHOUS.	NO ELECTROPHORESIS DONE.	182.5	74	3.4	27	220	52	3.5	30
		D			247.2		12.4		430		11.6	
19C	6 days	S*			—	—	—	—	—	—	—	—
		D			178.		14.9		320		18.4	
19G	6 days	S*			48.8	28	3.5	23	0	0	—	—
		D			175.		15.3		270		11.7	

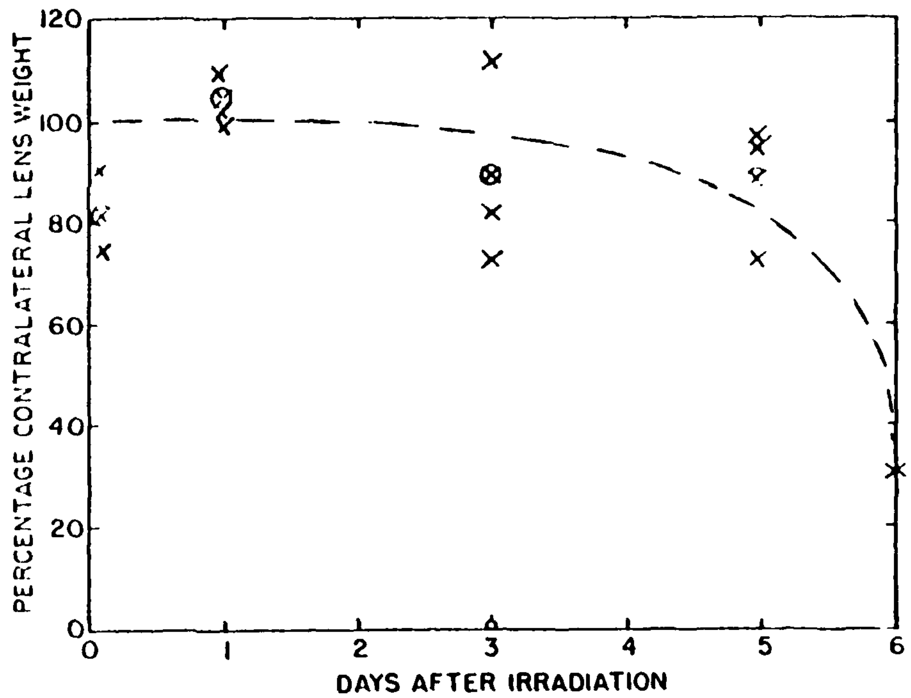


Figure IV-9 The weight of lenses from weanling rabbit eyes irradiated with the CO_2 laser (1.5 watts, 90 seconds, 8 *mm* diameter) as a *function* of time following exposure. Each data point (x) represents the lens weight taken from Table IV-1 expressed as a percentage of that of the contralateral control eye. The circles represent average values for each time group. It appears that there was no marked drop in lens weight until between the fifth and sixth days post irradiation.

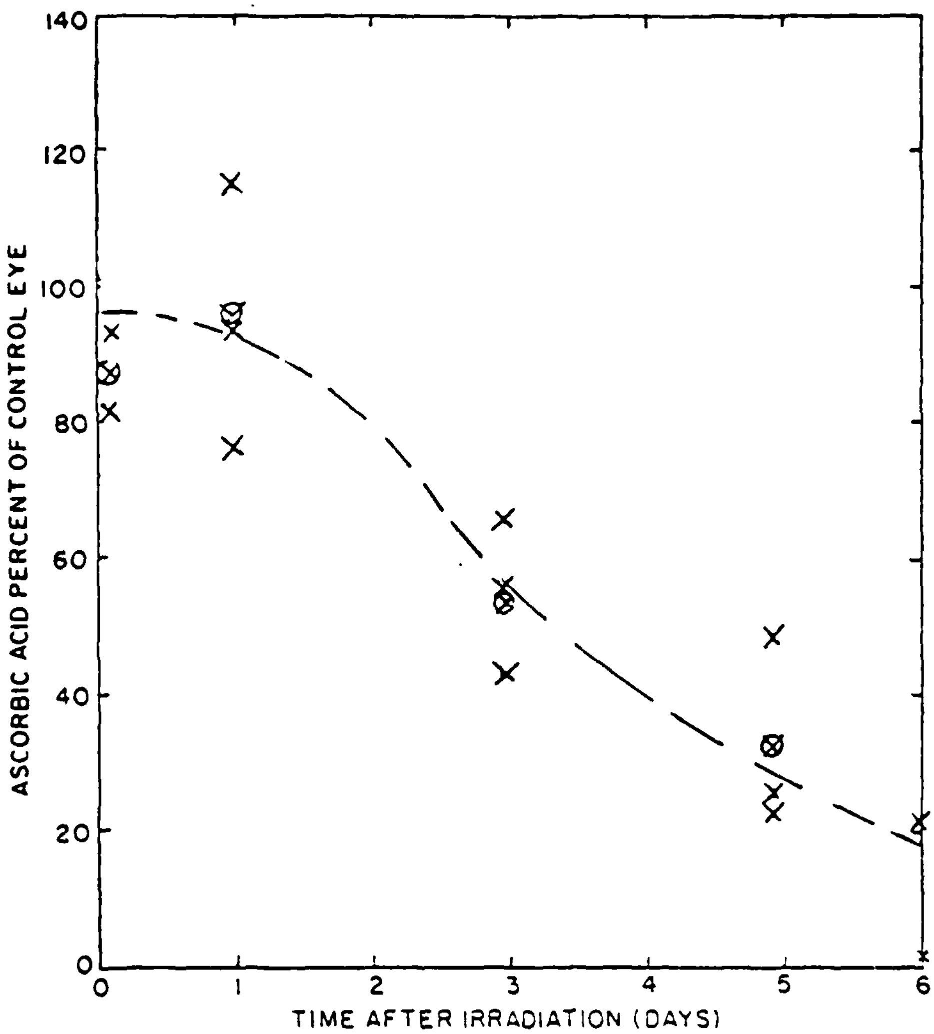


Figure IV-10 Relative ascorbic acid (AsA) concentration in weanling rabbit lenses as a function of time following CO_2 laser irradiation (1.5 watts, 90 seconds) of the cornea (5mm diameter area). Each data point (x) represents the lenticular AsA concentration (Table IV-1) expressed as a percentage of the concentration of the contralateral control lens. Each circled x is the average percentage value for each time group. The relative concentration of lenticular AsA appears to have decreased considerably between one and three days post irradiation, and continued to decrease after the third day post-irradiation.

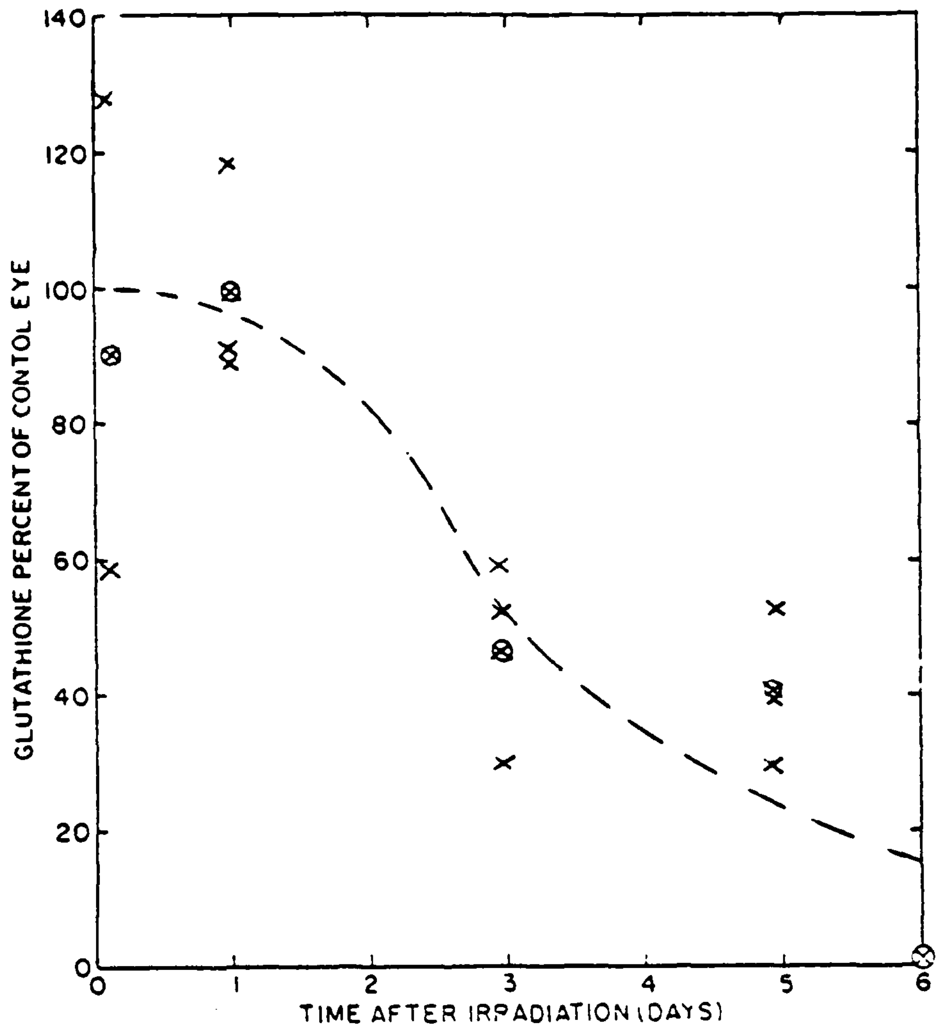


Figure IV-11 Relative reduced glutathione (GSH) concentration in weanling rabbit lenses as a function of time following CO_2 laser irradiation (1.5 watts, 90 seconds) of the cornea (6 mm diameter area). Each data point (x) represents the lenticular GSH concentration as a percentage of the contralateral control lens. Each encircled x represents the average percentage value for each time group. The decrement does not appear to have been significant during the first days post irradiation. Between one and three days there was a significant drop. The relative GSH concentration was reduced to an insignificant value by six days post irradiation.

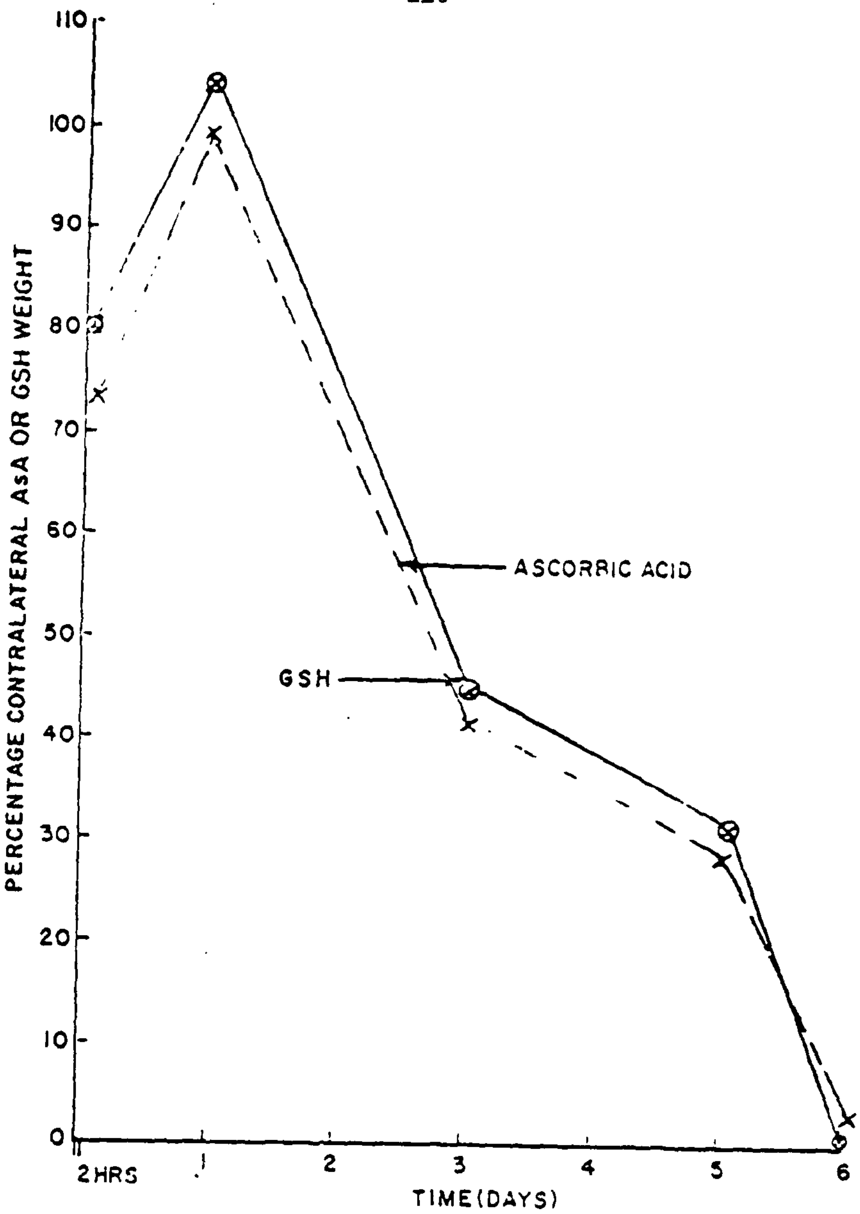


Figure IV-12 The relative total weights of lenticular ascorbic acid (AsA) and of reduced glutathione (GSH) following CO_2 laser irradiation (1.5 watts, 90 seconds) of the cornea (8mm diameter area) of weanling rabbits, as a function of time following exposure. Each data point is the average of the percentage of either the AsA or the GSH weight in the contralateral control lens. The values remained essentially constant during the first day following irradiation. There was a marked decrease between one and three days post irradiation. The total weight of both compounds was very low six days post irradiation.

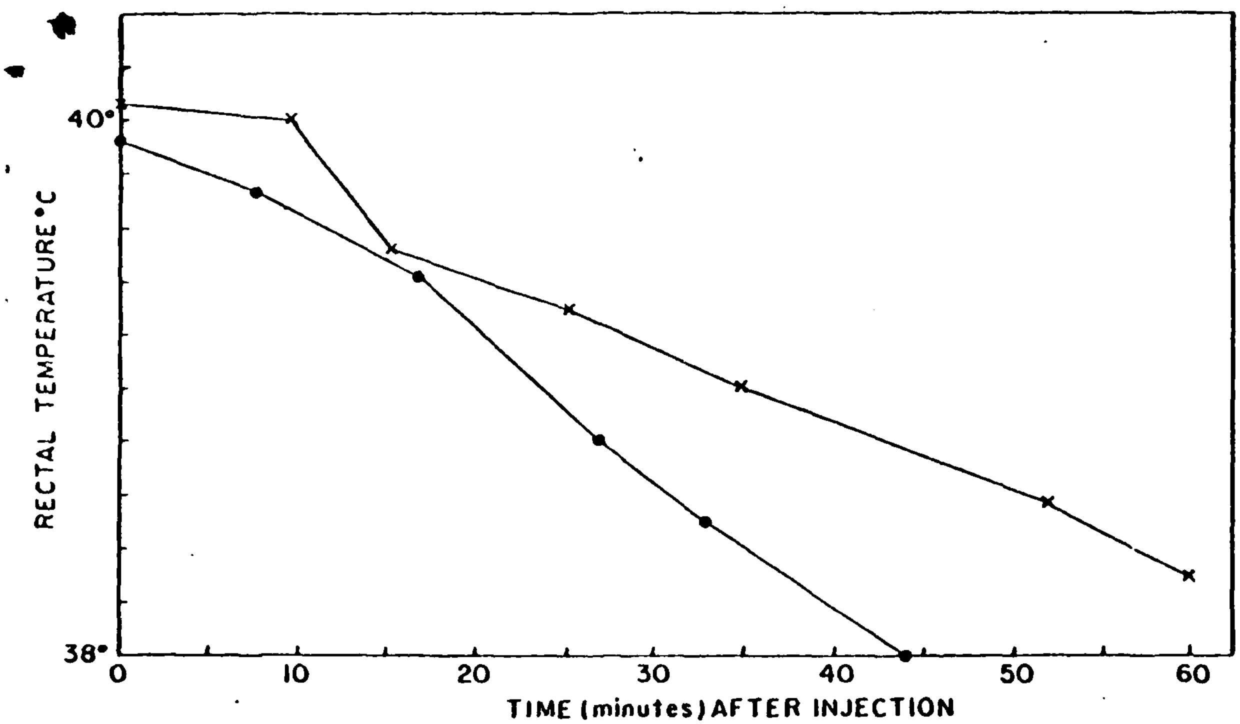


Figure IV-13 Effect of anesthesia--Urethane and Innovar Vet—on the rabbit rectal temperature as a function of time post injection. The rectal temperature decreased with time at a rate of about 2°C/hour during this period.

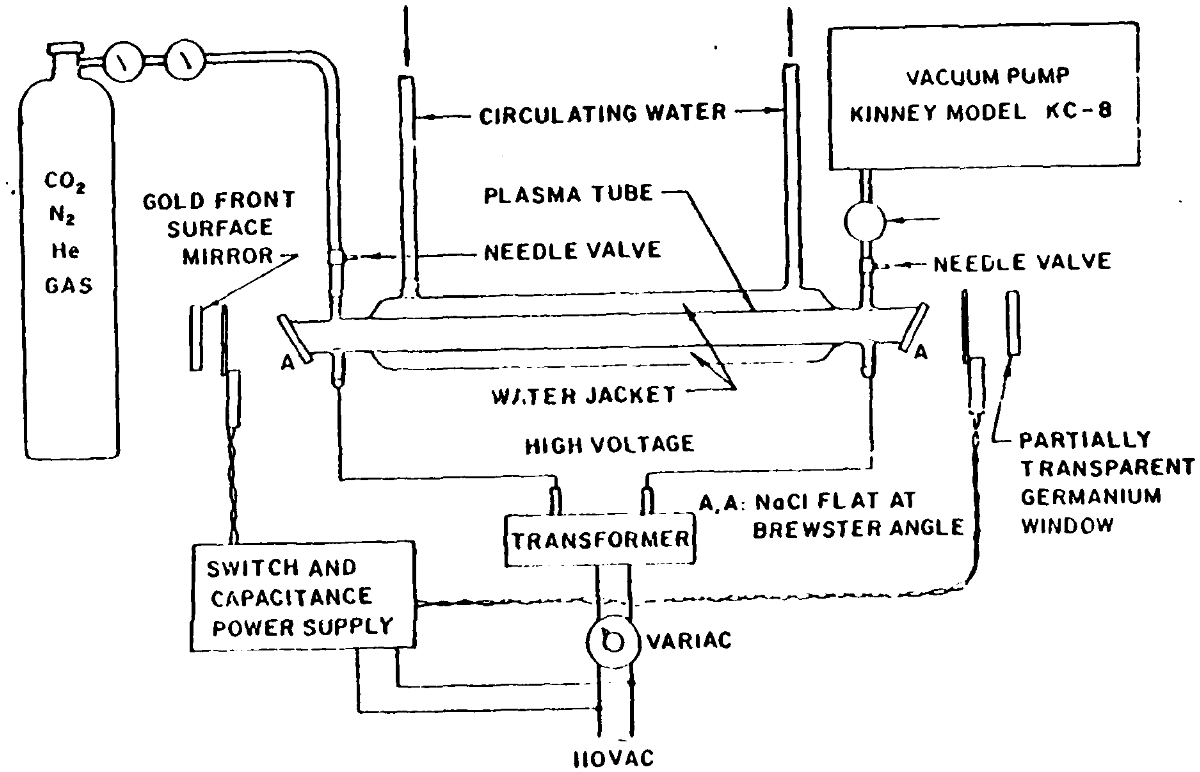


Figure A-1-1 Diagrammatic representation of the CO_2 laser and associated ancillary equipment used in this study.

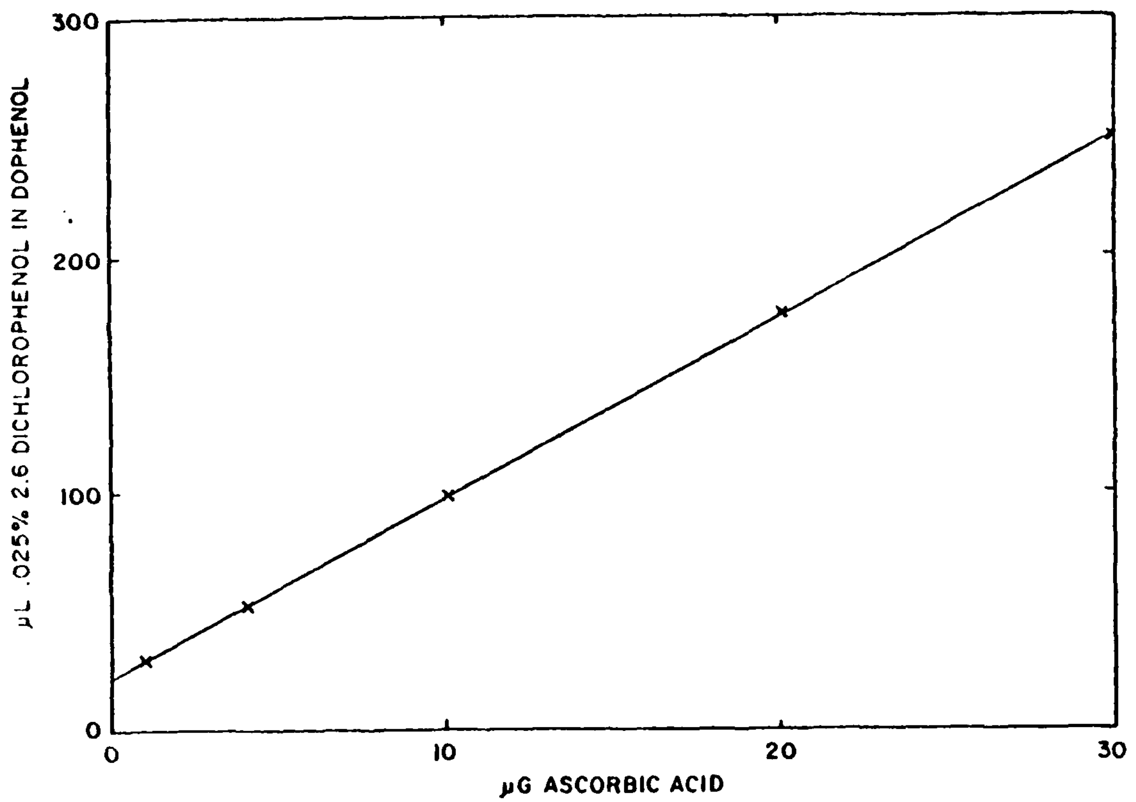


Figure A-3-1 A representation standard curve for ascorbic acid (AsA). The visual method of Roe (1954) using 2,6-dichlorophenolindophenol was employed. The curve was linear but did not pass through origin.

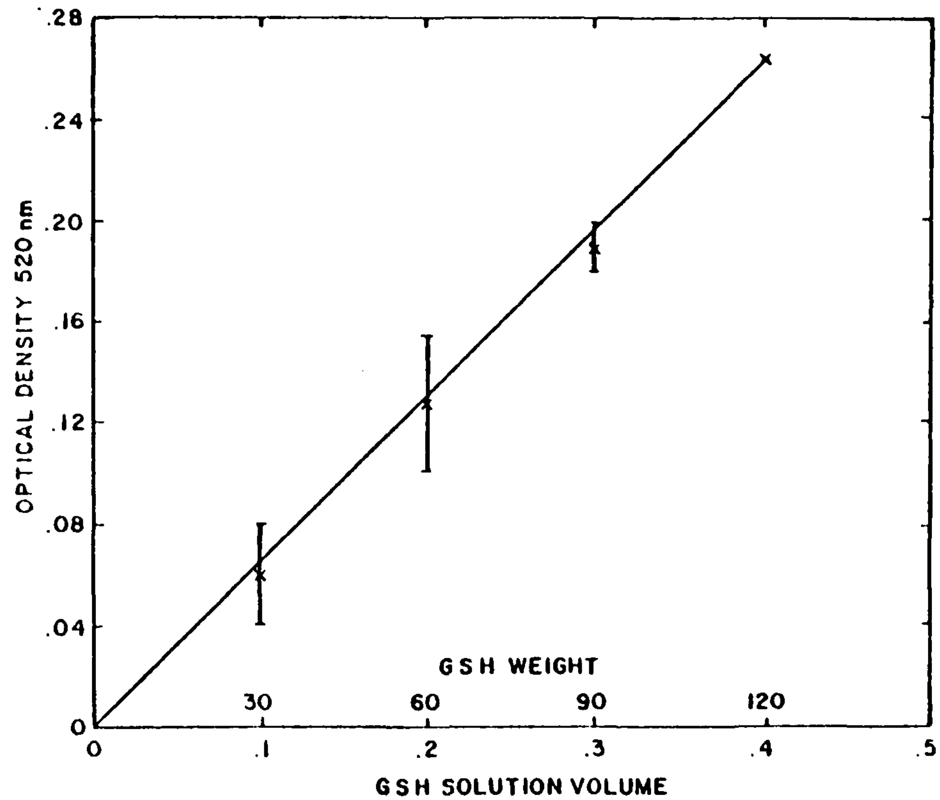


Figure A-4-1 A composite curve for reduced glutathione (GSH). The spectrophotometric method of Grunert and Phillips (1951) was employed. The data points represent the average values from a series of separate standard curve determinations; the error bars are one standard deviation. The curve was not fitted statistically.

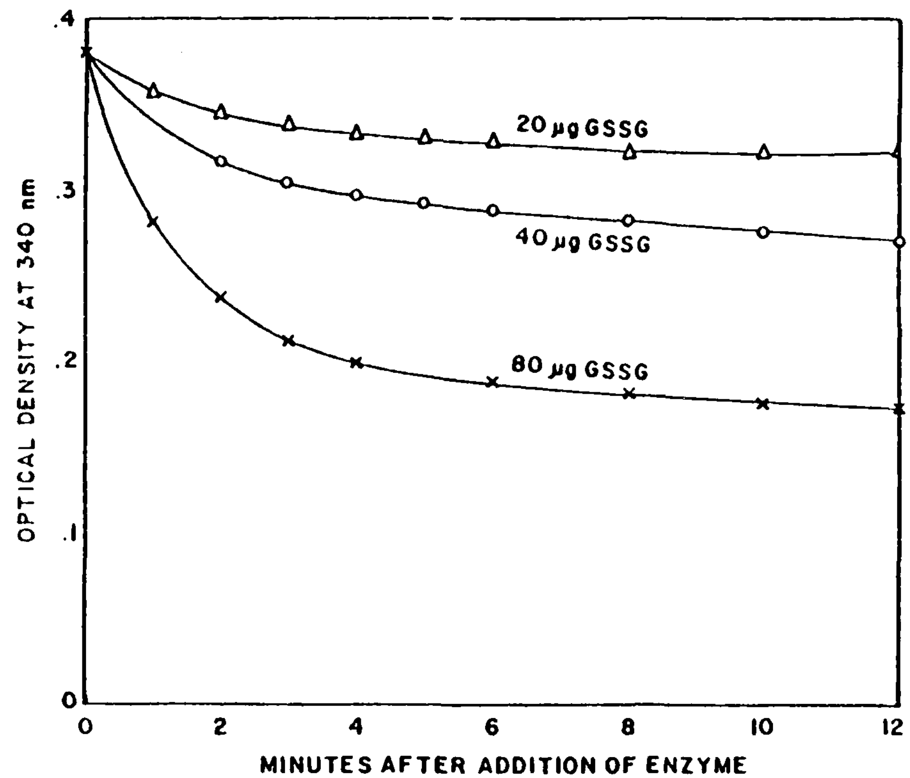


Figure A-5-1 Reaction rates for the reduction of three different concentrations of oxidized glutathione (GSSG) by glutathione reductase in the presence of reduced NADP. A modified method of Bergmeyer (1963) was employed. The change in optical density at 340 nm is plotted versus time. The three essentially exponential decay curves were obtained using identical concentrations of glutathione reductase and $NADPH_2$ for three different substrate concentrations.

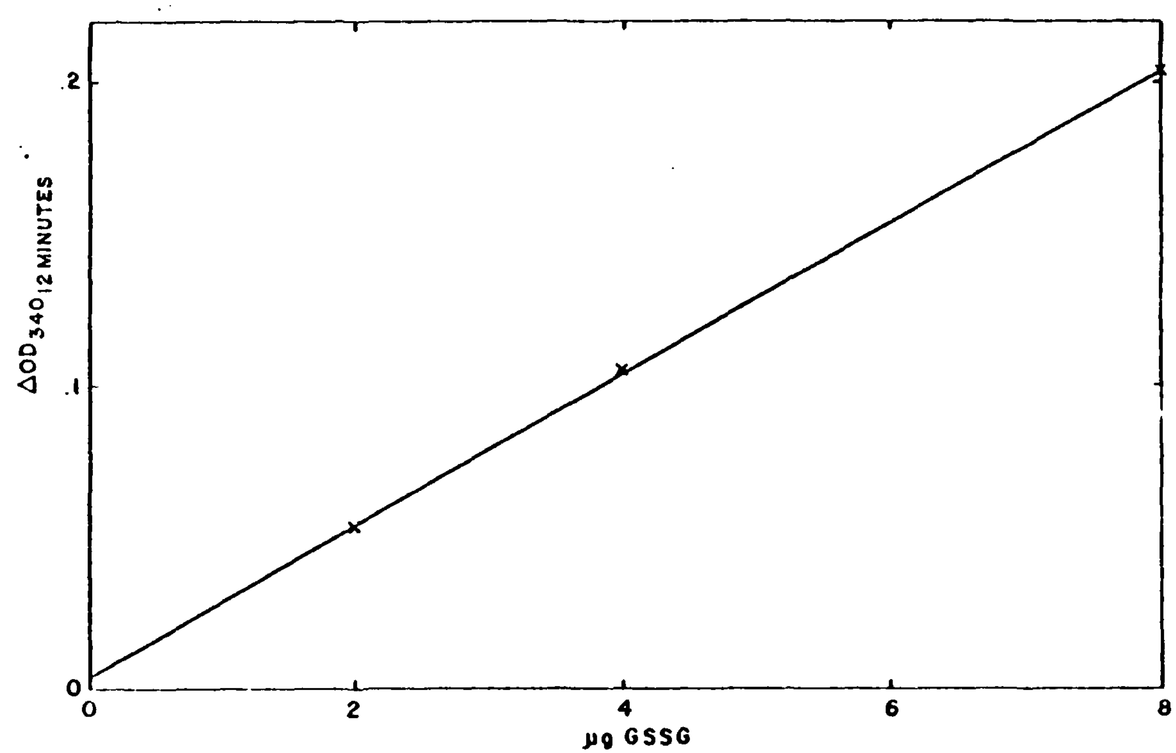


Figure A-5-2 Standard curve for oxidized glutathione (GSSG) made by utilizing the enzymatic method of Bergmeyer (1963). The graph is plotted as a change in optical density at 340 nm for the initial 12 minutes of reaction versus concentration of GSSG in micrograms. (The straight line is not statistically fitted to the data points.)

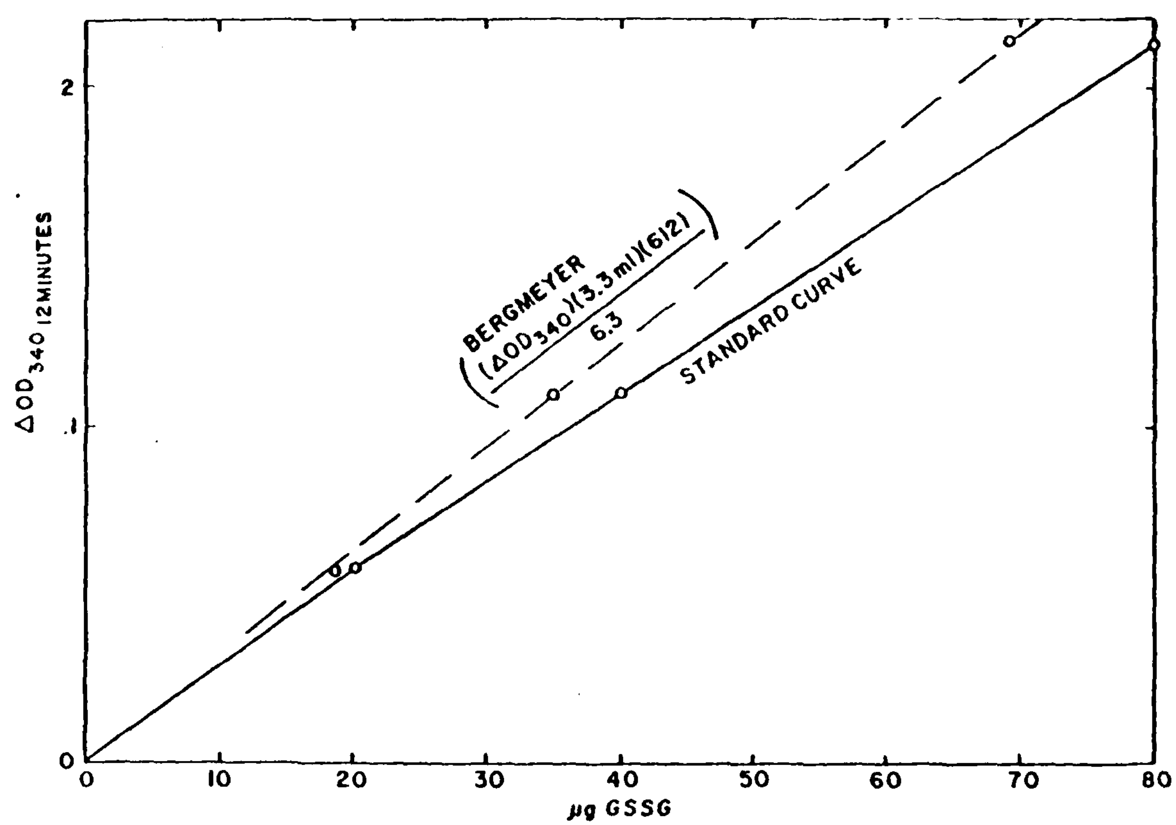


Figure A-5-3 Comparison of standard curve obtained experimentally with curve obtained from equation by Bergmeyer.

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PUBLICATIONS

A. Laser-Related

1. Fine, S., Klein, E., Scott, R.E., Aaronson, C. and Donoghue, J. "Biological Effects of Laser Radiation," Second Boston Laser Conference, August 1, 1963.

2. Fine, S., Maiman, T.H., Klein, W. and Scott, R.E. "Biological Effects of High Peak Power Radiation," Life Sciences, 3:309-322, March, 1964.

3. Fine, S. and Klein, E. "Effects of Pulsed Laser Irradiation of the Forehead in Mice," Life Sciences, 3:199-207, March, 1964.

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58. Cohen, E. and Fine, S. "In Vitro Effects of Laser Irradiation on Human Gamma Globulin," Federation Proceedings, 27:1, 473, March-April, 1968.

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61. Fine, B.S., Fine, S., Feigen, L. and MacKeen, D. "Corneal Injury Threshold to Carbon Dioxide Laser Irradiation," Vol. 66, No. 1, pp. 1-15, July, 1968, American Journal of Ophthalmology.

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63. MacKeen, D., Fine, S. and Klein, E. "Toxic and Explosive Hazards Associated with Lasers," Laser Focus, pp. 47-49, October, 1968.

64. Fine, B.S., Berkow, J.W., Fine, S. "Corneal Calcification," Science, Vol. 162, pp. 129-130, October 4, 1968.

65. Hansen, W.P. and Fine, S. "Application of Thermal Models to Retinal Threshold Injury," presented at Laser Industry Association meeting, October 24-26, 1968, published in Proceedings of the Laser Industry Association Convention, 1968.

66. Bock, F., Laor, Y., Fine, S. and Klein, E. "Exploration of Potential Carcinogenic Effects of Pulsed Laser Radiation," presented at Laser Industry Association meeting, October 24-26, 1968, published in Proceedings of the Laser Industry Association Convention, 1968.

67. Fine, S., MacKeen, D., Feigen, L. and Fine, B. "Anterior Chamber Measurements on CO_2 Laser Corneal Irradiation," Proceedings of the Annual Conference on Engineering in Medicine and Biology, Vol. 10, p. 6, November 4, 1968.

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72. Fine, S. and Klein, E. "Lasers in Biology and Medicine," Laser Focus, pp. 28-36, July, 1969.

73. Fine, S., Bushor, W. and Cos, M. editors. Proceedings of the Laser Industry Association Meeting, October 24-26, 1968.

74. Fine, S. and Klein, E. "Lasers in Biology and Medicine" published in Development in Laser Technology, Society of Photo-Optical Instrumentation Engineers.

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77. Klein, E., Laor, Y. and Fine, S. "Interaction of Laser Radiation with the Skin," Abstract-Laser Journal, Vol. 2, No. 1, January-February, 1970.

78. Fine, S., MacKeen, D., Berkow, J. and Fine, B.S. "Biological Studies with Laser Protective Materials," American Journal of Ophthalmology, Vol. 71, No. 4, April, 1971.

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81. Fine, S. and Hansen, W.P. "Optical Second Harmonic Generation in Biological Systems," Applied Optics, October, 1971.

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84. MacKeen, D., Cohen, J., and Fine, S. "Simultaneous Corneal Surface and Anterior Chamber Temperature Measurements on CO_2 Laser Irradiation," Federation Proceedings, Vol. 33, No. 3, Pt. 1, March, 1974. (Abstract.)

85. MacKeen, D.L., Szabo, G., and Fine, S. "The Effects of UV Laser Radiation at 325 nm on the Skin," The Yale Journal of Medicine, 1973 (Abstract).

B. Non-Laser Related

In addition, credit was given to non-laser related studies, in which the principal investigator and his associate were involved. A number of these were listed in the annual progress reports; several are listed below.

86. Fine, S., Klein, E., R.E., Hainish, H. and Aaronson, C. "Bio-Engineering in the Biological Sciences," IEEE Student Journal, January, 1964, 1:33-39.

87. Litwin, S.B., Cohen, J., Fine, S. and Aaron, A. "Rupture and Tensile Strength Measurements of Fresh and Teated Canine Aortic Tissue," Proceedings of the Annual Conference on Engineering in Medicine and Biology, Vol. 10, p. 44, November 4, 1968.

88. Aaron, A., Litwin, S.B., Fine, S. and Sillin, L. "Pressure and Flow Relations in Canine Aortic-Pulmonary Shunts," presented at Second International Conference on Medical Physics, Boston, Massachusetts, August, 1969, published in Abstracts of Conference.

89. Aaron, A., Litwin, S.B., Fine, S. and Rosenthal, A. "Determination of Cardiac Output by Dye Dilution," in Proceedings of the 23rd Annual Conference on Engineering in Medicine and Biology, Vol. 12, 1970.

90. Cohen, J., Litwin, S.B., Aaron, A. and Fine, S. "The Rupture Force and Tensile Strength of Canine Aortic Tissue," J. Surg. Research, December, 1972.

91. Litwin, S.B., Cohen, J. and Fine, S. "Effects of Sterilization and Preservation on the Rupture Force and Tensile Strength of Canine Aortic Tissue," J. Surg. Research, 1973.

C. Laser-Related Presentations as Invited Lecturer, Pertinent to Contract to Which Credit Was Given

1. Conference on Biological Effects of Laser Radiation, Washington, D.C. - Sponsored by U.S. Army Medical Research and Development Command, 1964

2. Conference on Lasers, New York Academy of Sciences, 1964

3. Gordon Research Conference on Biological Effects of Laser Radiation, 1965

4. Conference on the Biological Effects of Lasers, National Institutes of Health, Bethesda, Maryland, October 4-5, 1965

5. Gordon Research Conference on Biological Effects of Laser Radiation, 1966

6. Bell Telephone Laboratories - invited lecturer, 1966

7. The Martin Company - symposium on Biological Effects of Laser Radiation, 1966

8. Boston Medical Physics Society - Lecturer on Biophysical Studies with Laser Radiation, 1966

9. Seminar on Biological Effects of Laser Radiation, University of Texas, Austin, 1966

10. Conference on Development of Lasers in the Biological Sciences, Veterans Administration, Department of Medicine and Surgery, Washington, D.C., August 5, 1966

11. Presentation before the Physicians of the Association of American Railroads, Montreal, June 4, 1967

12. Gordon Research Conference on Biological Effects of Laser Radiation, 1967 (Session Chairman)

13. American College of Obstetrics - District I Meeting - Invited Participant - Biological Studies on Laser Radiation, October 1967

14. Invited Lecturer on Lasers - P.R. Mallory & Co. - Laboratory for Physical Sciences - Biological Effects of Laser Radiation, February, 1968

15. Brookhaven National Laboratory, Upton, New York "Biophysical Effects of Laser Radiation", May, 1968

16. New England Chapter Health Physics Society, "Biological Effects and Hazards of Laser Radiation", May, 1968

17. Case Western Reserve University, Cleveland, Ohio, Summer course on Laser Technology and Applications, presented lecture "Lasers in Biology and Medicine," July, 1968

18. G-APURSI Symposium (International Antenna and Propagation Symposium) "Electromagnetic Waves (Lasers) for Biological and Medical Applications", September, 1968

19. Rutgers University, New Brunswick, New Jersey, Participant in "Evaluation of Laser Hazards Course", October, 1968

20. S. Fine - "Biological Studies Relating to Laser Irradiation, Particulary with Respect to the Eye", Howe Laboratories, Massachusetts Eye and Ear Infirmary, Harvard Medical School, December, 1968

21. S. Fine - "Control of Laser Hazards and Management of Accidents", National Center for Radiological Health, U.S. Department of Health, Education and Welfare, Rockville, Maryland, February, 1969

22. S. Fine - "The Application of Lasers to Biology and Medicine," Conference on Trends and Directions in Biological Sciences of the Thirteen Colleges Curriculum Program Biology Teachers, Clark College, Atlanta, Georgia, March, 1969

23. S. Fine, Participation in Skin Laser Workshop, Second International Laser Safety Conference, Cincinnati, Ohio, March, 1969

24. S. Fine, Lasers--Characteristics, Use, Hazards and Biological Effects, Seminar Series, Environmental Health Engineering and Science, Graduate School of Engineering, Northeastern University, March, 1969

25. S. Fine, Lasers--Characteristics and Uses in Biology and Medicine, Surgical Seminar Series, Boston University School of Medicine, March, 1969

26. S. Fine, Use of Lasers in Biology and Medicine, Laser Applications Course, Washington University, St. Louis, Missouri, May, 1969

27. E. Klein, and S. Fine, Tissue and Cell Effects of Laser Radiation--Gordon Research Conference on Lasers in Medicine and Biology, June, 1969

28. S. Fine, "Lasers in Biomedicine," I.E.E.E. Student Branch, Northeastern University, July, 1969

29. S. Fine, "Biological Hazards and Effects of Laser Radiation," in course on Fundamentals of Non-Ionizing Radiation Protection, Northeastern Radiological Health Laboratory, August, 1969

30. S. Fine, Lasers in Industry, Associated Hazards and Protection, National Safety Congress, Chicago, Illinois, October 28, 1970

31. S. Fine, Non-invasive Testing in Medicine, I.E.E.E. group on Engineering in Biology and Medicine, Boston, Massachusetts, November, 1970

32. S. Fine, Uses and Hazards of Laser Radiation in Industry and in Atmospheric Pollution Studies, 24th AMA Clinical Convention, Boston, Massachusetts, November 30, 1970

33. S. Fine, "Bioengineering", Massachusetts Epsilon Chapter, Tau Beta Pi (Northeastern University), September, 1969

34. S. Fine, "Laser Biology", in Laser Fundamentals and Applications course, Polytechnic Institute of Brooklyn Graduate Center, September, 1969

35. S. Fine and E. Klein, "Biological Effects of Laser Radiation," the Theobald Smith Society, New Jersey, October, 1969

36. S. Fine, "Lasers--Biological Effects and Medical Applications," Society of Photo-optical Instrumentation Engineers Meeting, co-sponsored by the University of Rochester Institute of Optics, Rochester, New York, November, 1969

37. S. Fine, Session Chairman, Laser and Ultraviolet Contributed Papers, Fourth Annual Midyear Topical Symposium, Health Physics Society Meeting, Louisville, Kentucky, January 28-30, 1970

38. S. Fine, Lasers in Industry, Associated Hazards and Protection, National Safety Congress, Chicago, Illinois, October 28, 1970

39. S. Fine, Non-invasive Testing in Medicine, I.E.E.E. group on Engineering in Biology and Medicine, Boston, Massachusetts, November, 1970

40. S. Fine, Uses and Hazards of Laser Radiation in Industry and in Atmospheric Pollution Studies, 24th AMA Clinical Convention, Boston, Massachusetts, November 30, 1970

41. S. Fine, "Lasers in Biology and Medicine," in course on Lasers and Optics for Applications, Massachusetts Institute of Technology, Cambridge, Massachusetts, July 30, 1971

42. S. Fine, Guest Lecturer in Graduate Course 2.77, "Biological Effects and Medical Applications on Non-Ionizing Radiation," Fall, 1971, Massachusetts Institute of Technology

43. S. Fine, "Medical Applications, Research and Safety," Boston Section I.E.E.E. 1972 Lecture Series, February, 1972

44. S. Fine, "Lasers in Biology and Medicine," a course on lasers and optics for application, M.I.T., July, 1972

45. S. Fine, "Biological Effects and Medical Applications on Non-Ionizing Radiation," guest lecturer for several sessions in graduate course 2.77, M.I.T., 1972-1973

46. S. Fine, "Biological Effects and Medical Applications of Non-Ionizing Radiation," guest lecturer in graduate course at M.I.T., 1973-9974

47. S. Fine, lectured at Raytheon Research Laboratory on Electrical Hazards and Emergency Management of Accidents, 1974

48. S. Fine, "Biological Effects and Medical Applications of Non-Ionizing Radiation", guest lecturer in summer session course, M.I.T., July, 1975

Most other conferences in which abstracts or papers were published are included in the preceding bibliography.

D. Laser-Related Conference Organization and Planning; Pertinent to Contract

1. Boston Laser Conference, 1963

2. Boston Laser Conference, 1964

3. Institute of Electrical and Electronics Engineers - Member, NEREM Program Committee, 1965

4. Institute of Electrical and Electronics Engineers - Member, NEREM Program Committee, 1966

5. American Association for the Advancement of Science - Session Organizer and Chairman of Session on Biological Effects of Laser Radiation, 1966

6. Laser Industry Association Convention, October 24-26, 1968

7. Course on "Fundamentals of Laser Radiation Protection" given to personnel of U.S. Department of Health, Education and Welfare, 1968

8. Member, program planning committee on seminar series in applications of physical chemical techniques in Biology and Medicine, EMB, IEEE, Boston Section, 1969

9. Laser Industry Association Meeting - Los Angeles, California, October 20-22, 1969

10. Electro-Optical System Design Conference, September 22-24, 1970, New York Coliseum. Planning of sessions, session organization and chairman.

11. Major participant in the organization, planning, and instruction of personnel, and field work related to the first major survey on lasers and laser devices in the United States which was carried out be the State of Massachusetts and Occupational Health and Radiological Health, H.E.W., 1968.

PERSONNEL RECEIVING CONTRACT SUPPORT AND GRADUATE DEGREES OBTAINED

Arnold Aaron	Ph.D. in Engineering
Charles Aaronson	M.S. in Engineering
John Campbell	M.A. in Psychology
John Caron	M.S. in Engineering
Joel Cohen	M.S. in Biology
John Donoghue	M.S. in Engineering
Larry Feigen	M.S. in Physics
James Forman	M.S. in Engineering
Peter Hansen	Ph.D. in Engineering
Karl Hergenrother	Ph.D. in Engineering
Donald MacKeen	Ph.D. in Biology

Note: The above individuals were supported in full or in part for contract related work while carrying out graduate work. In some cases, the support was minimal.

OTHER NON-GRADUATE DEGREE PERSONNEL RECEIVING SUPPORT INCLUDE:

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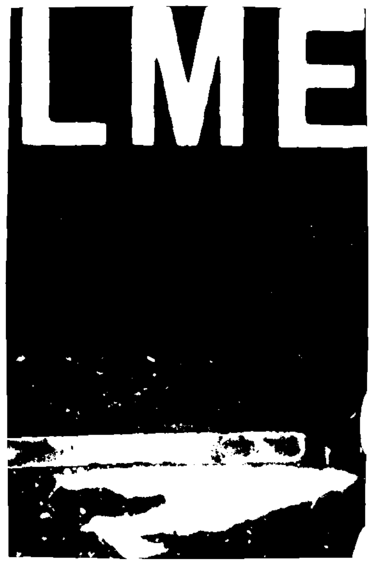
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